# 'MALARIAL PARASITE INDUCED STRUCTURAL ALTERATIONS IN MEMBRANE PROTEINS OF THE INFECTED RED CELLS'

A DISSERTATION SUBMITTED
TO THE
ALIGARH MUSLIM UNIVERSITY, ALIGARH
FOR THE DEGREE OF MASTER OF PHILOSOPHY
IN
BIOCHEMISTRY

BY

Chaudhary Anser Azim

M. Sc. (Biochem.)

MEMBRANE BIOLOGY DIVISION
CENTRAL DRUG RESEARCH INSTITUTE
LUCKNOW-226001

MARCH, 1989

Dedicated to

Late Umme-Habiba

and

Asma Azim

Telex : 0535-286
Telegram : CENDRUG
Phone : 32411-18 PABX

केन्द्रीय औषधि अनुसंधान संस्थान
चत्तर मंजिल, पोस्ट बाक्स नं० 173
लखनऊ 226001 (भारत)
CENTRAL DRUG RESEARCH INSTITUTE
Chattar Manzil, Post Box No. 173
LUCKNOW-226001 (INDIA)

No. Date

*Dr. C.M. Gupta, FASc, FNA*
*Head*
*Division of Membrane Biology*

## CERTIFICATE

*This is to certify that the work embodied in this thesis entitled 'Malarial Parasite Induced Structural Alterations in Membrane Proteins of the Infected Red Cells' has been carried out by Mr. Ch. Anser Azim, under my supervision.*

*He has fulfilled the requirements of the Aligarh Muslim University for the degree of Master of Philosophy in Biochemistry.*

*The work included in this thesis is original unless stated otherwise and has not been submitted for any other degree.*

(C.M. GUPTA)

## ACKNOWLEDGEMENTS

To my esteemed teacher, Dr. C.M. Gupta, FNA, Head, Membrane Biology Division, Central Drug Research Institute, Lucknow, I gratefully acknowledge an immeasurable debt for his skilful guidance, aesthetic suggestions and continued encouragement during the course of present investigation. He painstakingly, initiated me into a new field, with consumate skill and nurtured in me the ability for independent work. Whatever merits this dissertation possesses lie with him.

I am thankful to Dr. Masood Ahmad, Department of Biochemistry, Aligarh Muslim University, Aligarh, who as my teacher has always been happy to render me any help that I required.

I take this privilege to record my sincere thanks to Dr.M.M. Dhar, FNA, the former Director and Prof. B.N. Dhawan, FNA, the present Director, CDRI, for providing me the laboratory facilities.

I wish to express my deep sense of gratitude to Prof. A.M. Siddiqui, Prof. M. Saleemuddin and Prof. S.M. Hadi, who revealed me the fascinating field of Biochemistry.

Delighted, I feel to convey my sincere thanks to Drs.Ajai Kumar, Paritosh, Gokhale, Arun, Rashmi and Vinod for their constant cooperation and fruitful suggestions.

I wish to thank all my lab colleagues, Guddi, Ajai, Janardan Ranjana, Deka, Tanima, Anshu and Shome for their friendly advices and help.

Technical assistance and help of Mr. B.L. Srivastava and Mr. A.L. Vishkarma is gratefully acknowledged.

I am indebted to all my friends especially Drs Ashraf, Mashhood, Saroj, Alice and Mr. Faiyaz, Afaque and Alok Agarwal for their brotherly encouragements and help.

I wish to extend my deepest sense of gratitude to my parents, Baji, grandfather A.A. Siddiqui and mamoon A.M. Siddiqui for their unstinting support which bolstered me throughout the course of this investigation, particularly when the going became tough.

Words fail to come by which I can express my appreciation to my dearest friend Mashkoor, brother Ehsan and sister Nasrin whose love and encouragements were always with me.

The financial assistance from UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases is gratefully acknowledged.

Ch. Q. Azim

(CH. ANSER AZIM)

CONTENTS



*****

***

*

## ABBREVIATIONS

| | | |
|---|---|---|
| cAMP | ... | Cyclic adenosine-3'-5'monophosphate |
| ConA | ... | Concanavalin A |
| EDTA | ... | Ethylenedimaine-tetra-acetic acid |
| h | ... | Hour |
| kD | ... | Kilo dalton |
| L | ... | Litre |
| ml | ... | Millilitre |
| mg | ... | Milligram |
| mM | ... | Milli moles |
| min | ... | Minute |
| OD | ... | Optical density |
| PBS | ... | Phosphate buffered saline |
| rpm | ... | Rotations per minute |
| SDS | ... | Sodium dodecyl sulphate |
| ug | ... | Micrograms |
| uM | ... | Micro moles |

## PREFACE

Malaria is a serious impediment to economic development in the tropical countries. The causative agent of the disease is the malarial parasite which requires two hosts; a blood sucking mosquito, and a blood containing vertebrate. The development of parasite in vertebrate host commences with invasion of parenchymal cells of the mammalian liver or the endothelial cells of the bird by the sporozoite released into the blood stream, by an infected mosquito. These sporozoites develop and differentiate into merozoites (exo-erythrocytic schizogony), which are then released into the blood stream. These merozoites invade virgin red cells and undergo intracellular development through three stages viz. ring, trophozoite and schizont. The released merozoite re-invade fresh virgin red cells and the cycle continues.

Since, the red cells membrane deformability, structure and function are largely controlled by its association with its underlying membrane skeleton, it may be considered that to modify the host erythrocyte membrane structure and consequently the function, the intracellular parasite must first modify the membrane skeleton. A knowledge of these parasite-induced changes is essential for gaining a better insight into the host-parasite interactions. Therefore, studies on the parasite-induced molecular changes in the host erythrocyte membrane need to be undertaken to understand the mechanism that intracellular parasite employs for modifying the host erythrocyte membrane functions to its needs. Such studies may prove useful in designing new approaches to control malaria.

# REVIEW OF LITERATURE

## GENERAL STRUCTURE OF BIOLOGICAL MEMBRANES

The functional characteristics of all cellular and intracellular membranes are controlled by their chemical composition as well as by orientation of the component molecules in their structural frame work. The main chemical constituents present in all the biological membranes are lipids and (glyco) proteins. Since the membrane components have an essentially an amphiphillic character, they are forced by their duality to adopt a unique orientation with respect to the aqueous medium and form a highly organised structure (1). Phospholipids constitute the major portion of the membrane lipids and usually exist in bilayer configuration (2). The phospholipid bilayer serves as the basic permeability barrier as well as the structural matrix of all types of biological membranes. This bilayer in association with membrane proteins provides functional diversity and mechanical stability to the membranes.

One of the most prominent characteristics of phospholipids bilayers is their ability to undergo a reversible thermotropic phase transition (3) from fluid state (where phospholipid structure is highly disordered; $L_\alpha$ or liquid crystalline state) at high temperature to a gel state (where phospholipid structure is highly ordered; $L_\beta$, or gel crystalline state) at low temperature. The temperature at which such a transition occurs is commonly known as melting transition temperature Tm or Tc. Most of the biological membranes are present in liquid crystalline phase or fluid state. This may have been necessitated to accommodate membrane proteins

in the phospholipid bilayer and also for making the membrane deformable.

The gross arrangement of various membrane constituents in the bilayer matrix is best described by the 'Fluid Mosaic Model' (Fig. 1) of Singer and Nicolson (4). According to this model, every lipid and protein molecule is free to diffuse in the membrane plane (lateral diffusion). This model classifies membrane bound proteins into two categories 1) integral, and 2) peripheral proteins. Peripheral proteins reside on the membrane surface and associate with the membrane bilayer mainly by electrostatic and dipolar interactions, whereas the integral proteins remain partly embedded in the bilayer matrix and associate with membrane lipids by forces essentially derived from the hydrophobic effect (1). Integral proteins have further been classified in three categories, viz; ecto, endo and transmembrane, depending on their location in the membrane bilayer. Proteins which span the whole bilayer thickness are termed as transmembrane proteins (C- and N-terminals are exposed to the opposite surfaces) while proteins whose both C- and N-terminals are either exposed at the extracellular or intracellular sruface are termed as ecto and endo proteins respectively. This model was later modified by Nicolson (5), who suggested that lateral diffusion of some of the integral proteins is impeded by their association with peripheral proteins located at the cytoplasmic surface of the membrane (cytoskeletal proteins). The interactions between integral and cytoskeletal proteins are

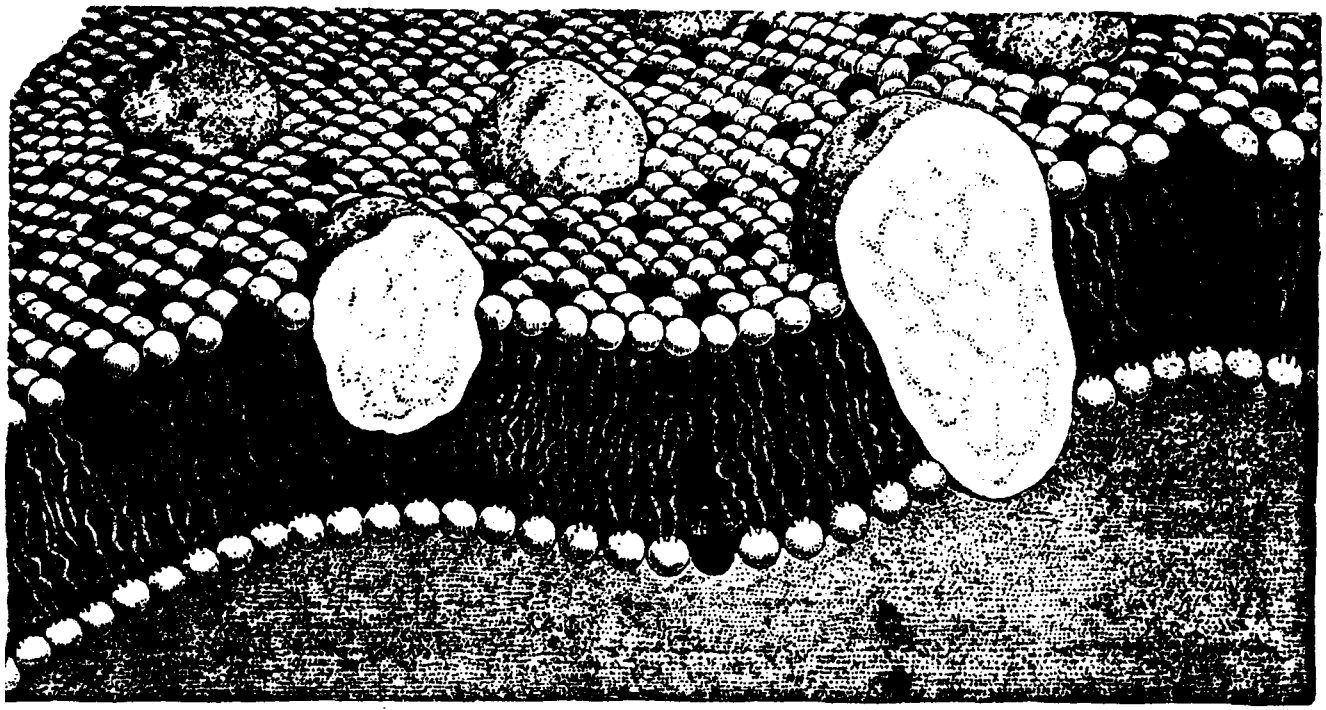


**Fig. 1:** Three dimensional fluid mosaic concept of membrane structure. The solid bodies represent the globular integral proteins which at long range are randomly distributed in the plane of the membrane. The open circles denote the ionic and polar head groups of the phospholipid molecules which make contact with water and wavy lines.

probably responisble for transmission of receptor mediated signals from the cell exterior to the cell interior (6), and control such important membrane events like fusion (7) and endocytosis (8).

Another important structural feature of biological membranes is that their constituents are asymmetrically distributed in the two monolayers which are described below.

**Lipid Asymmetry:**

After Danielli-Davson's discovery that phospholipids when dispersed in water from bilayer (2), another major breakthrough in membrane research has been that of Bretscher's finding that biological membranes are vectorial structures (9), that is their components are asymmetrically distributed in the bilayer. Every copy of a given protein in the membrane has the same orientation, whereas almost every type of lipid is unequally distributed in the two surfaces of the membrane bilayer.

A large number of studies on localization of membrane phospholipids in the two surfaces of different types of biological membranes have been carried out. The phospholipid distributions in membranes have been determined mainly by employing (a) chemical labelling reagent (b) enzymatic probes (c) phospholipid exchange proteins and (d) immunological techniques.

**Asymmetry of Proteins:**

There are reports that biological membrane proteins are

not symmetrically distributed or to be unexposed on either surface.

**Asymmetry of Carbohydrates:**

Carbohydrates are found associated only with extracellular portions of the membrane components which is supported by the observation that secreted proteins are generally glycosylated while cytoplasmic proteins are not glycosylated.

**THE RED CELL MEMBRANE**

The red cell membrane is composed of a bilayer of lipids into which proteins are inserted, and this is laminated onto an underlying protein lattice network, the cytoskeleton (10,11).

Here, first the red cell membrane proteins are described and then the red cell membrane phospholipids.

**Membrane Proteins:**

When membranes are dissolved in excess of sodium dodecyl sulphate (SDS) the proteins get separated both from the lipids and from one another. Electrophoretic separation in a SDS-polyacrylamide gel resolves some 40 constituents, of which 8-12 are the major polypeptides (Fig. 2).

Membrane proteins are divided into 2 major classes: integral and peripheral proteins. Integral membrane proteins penetrate or span the lipid bilayer and interact with the hydrophobic lipid core. Band 3, Band 4.5 and the glycophorins are the three major

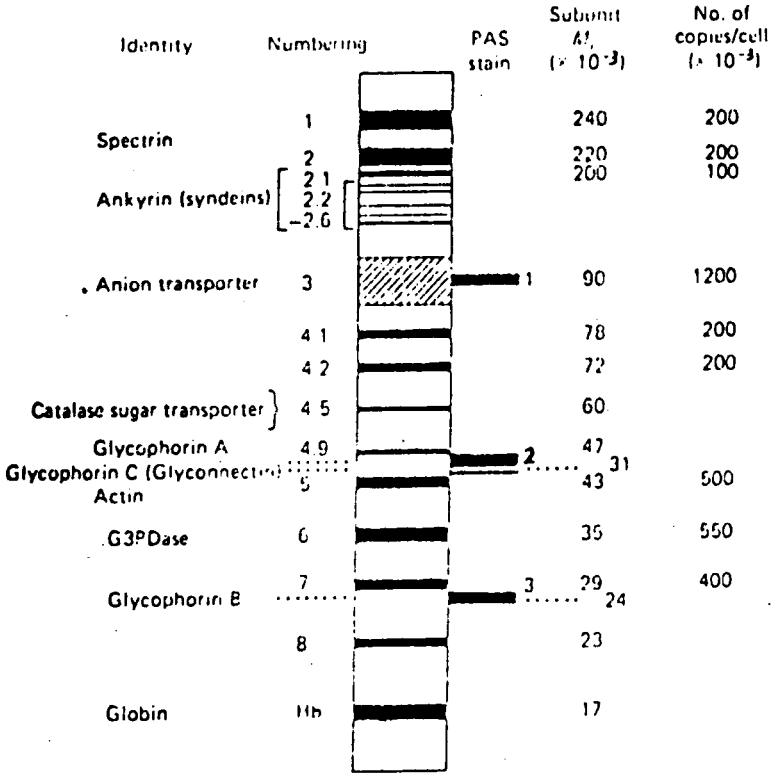



**Fig. 2:** A schematic representation of the major membrane proteins of the human red cell separated by SDS-PAGE. Gel banding patterns are shown after Coomassie blue or periodic acid-Schiff's staining. The values for subunit molecular weight and abundance are based on Gratzer (115) and Goodman & Schiffer (116).

integral membrane proteins in red cells. These are glycoproteins and ofcourse, the glycophorins are comparatively more glycosylated than the other two. The glycosylated portion of these proteins is exclusively localized on the outer surface of the membrane bilayer. These proteins bear the various blood group antigens, lectin binding sites and other antigen recognition sites.

Peripheral proteins are not inserted into the bilayer and reside on the inner surface of the membrane. They are bound by the electrostatic forces to the membrane lipids and integral proteins. The major peripheral proteins, which consists mainly of spectrin (bands 1 and 2), actin (band 5), band (4.1) and enzyme glyceraldehyde-3 phosphate dehydrogenase located on the cytoplasmic side of the membrane, form a 2-dimensional cytoskeletal network (Fig. 3).

A brief review of the structure and function of the red cell membrane proteins is given below:

Spectrin:

Spectrin is the major component of the red cell cytoskeleton (70%) proteins. There are about 200,000 copies of spectrin per human red cell. It gets detached from the red cell membrane by low ionic strength extraction, suggesting that the attachment of this protein with membrane is electrostatic. It is a heterodimer, consisting of two polypeptides referred to as band 1-(240 kD) and band 2-(220 kD), they are also called as $\alpha$ and $\beta$ spectrins.

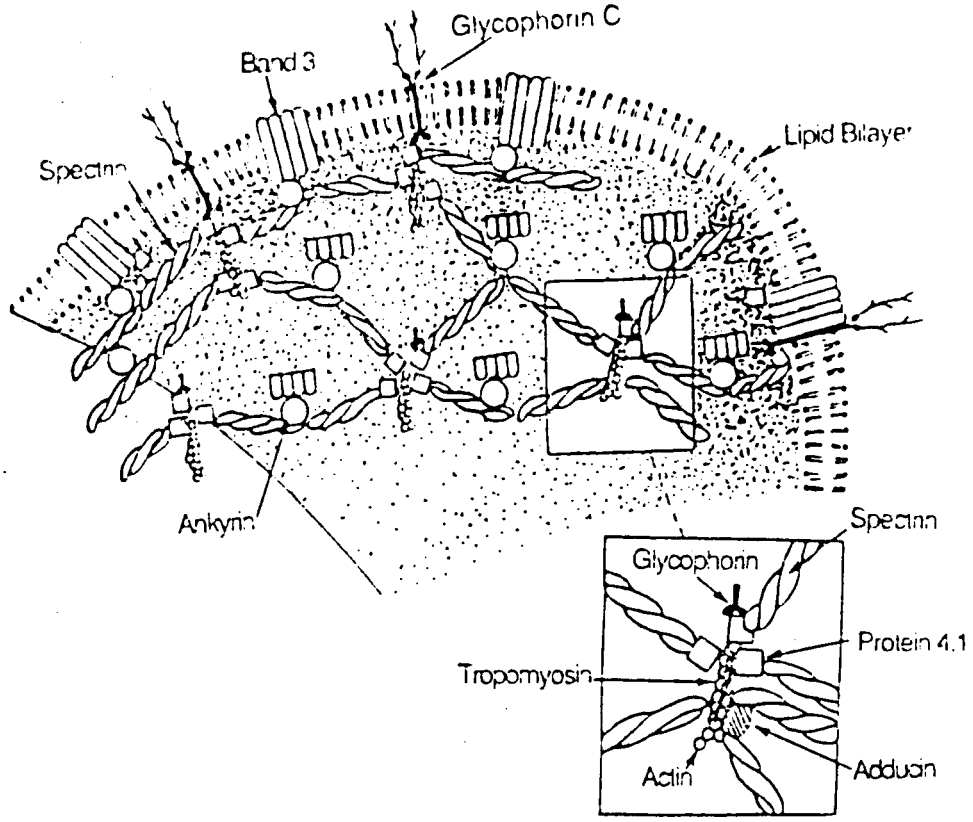



**Fig. 3:** Schematic model of the organization of proteins in the human erythrocyte membrane. From Gardner and Bennett (117).

The gene for $\alpha$ subunit lies on chromosome 1 and, while the $\beta$ subunit gene is on chromosome 14. The quaternary structure of the isolated spectrin is dependent on the mode of extraction. AT $4^{o}C$ the extracted protein is in tetrameric form while if extraction is performed at $37^{o}C$ the spectrin is obtained in tetrameric as well as dimeric form (12).

Spectrin dimer and tetramer are interconvertible and this conversion is temperature dependent. This is due to the high activation energy characterising the interaction (13). This is supported by the observation that the physiological amount of spectrin can be rebound to spectrin depleted vesicles only when tetramer is used (14), and only membrane skeletons containing tetramer are stable to mechanical stress (15).

Higher oligomers of spectrin can be formed in concentrated spectrin solutions incubated at $30^{o}C$ and small quantities of higher oligomers appear to be present in membranes (16). However, it has been suggested that these higher order oligomers are not necessary for the maintenance of the membrane cytoskeletal integrity.

Conformational characteristics of spectrin:

Circular Dichormism measurements (17, 18, 19) show that spectrin has a high-helical content (60-70%). The hydrodynamic properties of spectrin are those of a highly asymmetric molecule. The sedimentation coefficient for the spectrin dimer is 9.5S (for a globular protein of the same molecular weight it would be about

18S corresponding to a frictional ratio of 2.1). The solution properties of spectrin show a striking dependence on ionic strength beyond the range of known charge effects unrelated to conformational changes. The light scattering data of Elgsacter (20) has provided the most conclusive conformational characterization of salt dependent changes in spectrin. A substantial increase in the radius of gyration can be observed when the ionic strength is decreased (reaching 40 nm at 1 mM salt). This suggests a conformation which is capable of a large degree of flexibility. The above observations are supported by the proton magnetic resonance spectroscopy (21), which exhibited sharp peaks corresponding to a portion of the aliphatic side chains thus proving that these signals are coming from protons in an unrestricted environment. The electron rotory shadowing microscopic studies of Shotton et al (22) have been extremely helpful in resolving the structure of spectrin. It revealed that dimer is an elongated molecule some 97nm in length, the two chains lying side by side and loosely associated possibly coiled around each other and joined at both the ends. The spectrin is worm like rather flexible and bent molecule in solution. The tetramers consists of two dimers associated end to end. This association is evidently head to head since spectrin does not polymerize in a continuous isodesmic manner. Hexamers and higher order oligomers can be seen at low abundance in rotory shadowed preparations and their mode of formation is the head to head association. Spectrin is an acidic protein with an PI of about 5.0. The amino acid composition of the two subunits are remarkably similar and

are unusual in their high content of aspartic acid and glutamic acid. It is clear that the subunit is not derived from by post translational proteolysis. Digestion of spectrin by trypsin results in the appearance of a sequence of fragments as seen on SDS gels, which is consistent with a repeating structure within the molecule. Sequencing studies have confirmed the deduction of repeating units. A sequence repeat of 106 amino acid residues have been identified and is present in both the $\alpha$ and $\beta$ chains (23). Homology is variable between repeat sequences except at position 45 where tryptophan is invariably conserved. Spectrin contains four covalently bound phosphate groups which are located within a 10,000 dalton peptide of the terminal end of the subunit (24). It is phosphorylated by two cAMP independent kinases, one cytoplasmic, the other membrane bound. All four sites are equally prone to phosphorylation (24). Spectrin can also be phosphorylated in vitro by a cAMP dependent kinase, this has no physiological role.

Actin:

This is another major cytoskeleton protein, which is present in about 400,000-500,000 copies per cell and most of these molecules are associated with the membrane skeleton. The concentration of free actin in erythrocyte cytosol has been estimated at 15 ug/ml (25) which is close to the critical concentration of free actin filaments. Isolated erythrocyte actin has been found to be identical to actin from other cell types in that it polymerizes into extended

filaments, and activates myosin ATPase activity (26). However, there is one difference of erythrocyte actin is the primarily one isoform (beta) (25), while other cells have a mixture of actin isoforms which gives rise to a possibility that the erythrocyte actin isoform has specialized functional properties. The erythrocyte actin is assembled into defined short filaments containing on the average of 12-14 actin monomers. Direct evidence of an oligomeric form of actin has been provided by electron microscopy which reveals rather uniform structures of 7-8 nm in width and an average length of 33-37 nm which nucleate assembly of actin filaments (27).

Protein 4.1:

Protein 4.1 is present in about 200,000 copies per cell and is the major accessory protein associated with spectrin and actin in membrane skeletons and isolated functional complexes. About 80 percent of protein 4.1 remains associated with erythrocyte membranes following extraction of spectrin and actin by low ionic strength buffer, and nearly all of the protein 4.1 is recovered in membrane skeletons prepared by extraction of erythrocyte ghosts with nonionic detergent. Protein 4.1 has been purified either from low ionic strength extracted membranes (28), or from membrane skeletons following solubilization in 1M Tris (29). Protein 4.1 is a monomer in dilute solution, having a molecular weight of 78,000 daltons by sedimentation equilibrium measurements.

Protein 4.1 consists two polypeptides referred to as 4.1a and 4.1b that differ in apparent molecular weight by about 2,000

daltons. The difference in mobility on SDS-gels originates from the carboxy-terminal (Mr=22,000, 24,000)domains of 4.1, but probably is not due to a difference in primary sequence, but due to a post-translational modification that occurs during maturation and aging of erythrocytes. The lower molecular weight form 4.1b predominates in young erythrocytes while the higher molecular weight 4.1a is the major form in older cells (30). A notable feature of protein 4.1 is the large size of the mRNA which is 5.6 kilobases in length or three time the length required to encode for the protein. The genomic DNA coding for protein 4.1 is even more oversized with an estimated length of at least 40 kilobases (31). It is of interest that in avian erythrocytes and lens multiple forms of 4.1 are expressed with molecular weights up to 175,000 daltons and the pattern changes during erythroid differentiation. A single 4.1 gene thus is likely to produce multiple mRNAs by tissue-specific and developmentally regulated alternative splicing.

Ankyrin:

Ankyrin or syndein is a family of proteins of band 2.1 series. Band 2.1 has been characterized fully. It is an asymmetric molecule with a sedimentation coefficient of 6.9 S. Its molecular weight ranges from 200-210 kD. One hundred number of copies are present for $10^{-3}$ cells. It acts as a link between subunit of spectrin and integral protein band 3.

Band 4.2:

This is a peripheral protein of about 72 kD molecular weight. There are about 200,000 copies per red cell and accounts about

5% of the total membrane proteins. Its binding with the cytoplasmic domain of band 3 is reported (32).

Band 4.9:

Protein 4.9 is a 48,000 molecular weight polypeptide associated with spectrin-actin complexes that has been demonstrated to interact with actin filaments by in vitro assay (33). Band 4.9 is present in detergent extracted membrane skeleton and is partially extracted from membranes with low ionic strength buffer.

Protein 4.9 does not associate with spectrin alone, but does bind to and bundle actin filaments. Actin bundling activity does indicate that 4.9 has two actin binding sites.

Tropomyosin:

Erythrocyte tropomyosin is comprised of two polypeptides of Mr=29,000 and Mr=27,000 that are present as dimers in about 70,000-80,000 copies per cell (34). Erythrocyte tropomysin has been purified and demonstrated to possess a number of properties in common with other tropomyosin proteins including common antigenic sites, physical properties of an asymmetric dimer with a calculated molecular weight of 60,000 daltons, characteristic amino acid composition, isoelectric precipitation at pH 4.5 and heat stability. Erythrocyte tropomyosin associates with actin filaments in a highly cooperative fashion with a stoichiometry of one dimer per 6-7 actin monomers. Erythrocyte tropomyosin has been proposed to be associated with the actin filaments in the membrane skeletons in a

manner analogous to tropomyosin and actin in other systems with a tropomyosin dimer attached along each of the two grooves of the actin helix.

Myosin:

The first proof for the presence of myosin in red cells was reported by Kirpatrick and Sweeney in 1980 (35). About 6,000 copies of myosin are present per red cell. Myosin probably controls the shape changes in the erythrocyte as it passes through narrow capillaries and sinusoids. An association of myosin with integral membrane proteins has been shown, suggesting that myosin and actin could serve as secondary sites of linkage of the bilayer to the skeleton.

Glycophorins:

Glycophorin A, an 31 kD protein, is the major sialoglycoprotein of the red cell membrane, called as glycophorin A containing 60% carbohydrate and 40% protein. There are about 370,000 copies of glycophorin A/cell and it constitutes about 1-2% of the total membrane proteins by weight. In its monomeric form it is identified on PAS-stained gel as PAS-2 whereas in its dimeric form it is identified as PAS-1 (Fig. 2). It probably exists as a dimer in the membrane. The outermost portion of the protein molecule contains most of the sialic acid, the MN blood group antigens, binding sites for influenza virus and lectins such as phytohaemagglutinin

and wheat germ agglutinin (WGA). Glycophorin A is responsible for much of the cell's negative surface charge. Glycophorin B, an 24 kD glycoprotein contains 5-10% carbohydrate, constitutes 0.2-0.5% of the membrane protein, and corresponds to PAS-3 on SDS-PAGE (36). It carries the receptor for WGA, the N,S and s antigenic sites with the first 23 amino acid residues identical to glycophorin A from N,N cells.

En(a$^-$) red cells are deficient in glycophorin A whereas individuals who lack blood group antigens S and s ($S^-$ $s^-$) have decreased amount of glycophorin B. Despite this, no erythrocyte shape change function or life-span has been noted in these glycophorin deficient cells (37, 10).

Glycophorin C:

The N-terminal portion of this glycoprotein differs from that of glycophorins A and B. Glycophorin C migrates in the region of PAS-2 (Fig. 2 ). This protein has been called glycoconnectin since it may be associated with the cytoskeleton through attachment to Band 4.1 (38, 39).

Band 3:

The transport of anions across the red cell membrane is mediated by the major integral glycoprotein, band 3. The protein was designated Band 3 by Fairbanks et al (36) and has been comprehensively reviewed by Steck (40). It migrates as a broad

zone on SDS-polyacrylamide gel with a mobility which corresponds to a molecular weight between 90,000 and 110,000 daltons. The width of the zone has been attributed to a heterogenity of glyosylation, as it appears to have only a single type of peptide backbone. It represents about 25% of the total Coomassie-Blue staining material of the membranes, which corresponds to about $1.2 \times 10^6$ copies per cell.

Band 3 is obtained as a stable dimer when isolated in presence of nonionic detergents. There is some evidence that it exists in the membrane as a noncovalently linked tetramer although, the possibility that the two forms might exist in equilibrium cannot be dismissed.

A number of functions are served by band 3 in the membrane. First, and foremost, it helps in anion exchange (i.e. for $HCO_3^-$) across the red cell membrane. The transporter behaves as a classical membrane carrier. The transported ions interact with binding sites accounting for the saturation type kinetics, competition between substrates, anion specificity and action of competitive-inhibitors. Kinetic data are overwhelmingly in support of a ping-pong mechanism for anion exchange, in which the anion binds at one surface and is transported to the other. Another anion binds at the surface and is transported to the opposite side. The transport site is then available for another cycle. Band 3 helps in the transport of different halides, $HCO_3^-$, $PO_4^{-3}$, $SO_4^{-2}$ etc, at varying rates. It is also believed that band 3 facilitates the entry of water across

the red cell membrane (41). Band 3 can be cleared into two distinct fragments by trypsin or chymotrypsin treatment on the inner face of the membrane. The 42 kD cytoplasmic domain is completely water soluble and has binding sites for Hb, glycolytic enzymes, bands 2.1 and band 4.2. It plays no role in the transport activity of the membrane. The membrane spanning domain has a molecular weight of approximately 55 kD. It bears the anion exchange activity, as well as the carbohydrate moiety.

The sequence of the first 20 residues of the 42 kD cytoplasmic domain has been determined (42). The amino terminal region is extraordinarily acidic with 6 aspartate and 12 glutamate residues out of the first 33 amino acids. The region serves as the attachment site of all glycolytic enzymes. Near the 42 kD domain lies the binding site of ankyrin. The 55 kD membrane spanning domain is involved in the anion transport across the membrane. The positive charges on lysine residues present in this segment are probably essential for binding of anionic substrates(43).

Band 4.5:

The 4.5 polypeptide is an integral membrane protein responsible for monosaccharide transport by facilitated diffusion. It amounts to about 9.8±1.9% of the total membrane proteins (44) and about 124,000-194,000 copies are present per cell (45). It migrates as a broad band of 43 to 44 kD on SDS-PAGE gels due to heterogeneity caused by glycosylation. Carbohydrate constitutes about 15% of protein by weight (46).

**Interaction of the Membrane Skeleton with the Intrinsic Membrane Domain:**

Studies by Bennett and Branton (47) have revealed that spectrin binds with high affinity to inside out vesicles already depleted of spectrin. Binding of spectrin was inhibited when spectrin depleted inside out vesicles were treated with trypsin due to the release of 72 kD fragment. This 72 kD fragment was immunologically confirmed to be the ankyrin fragment and the membrane attachment site for spectrin. Binding studies also confirmed that band 2 attaches with ankyrin with an affinity constant of $4.3 \times 10^6$ (48). The 72 kD water soluble fragment has spectrin binding site while a 90 kD fragment has been reported to have the binding site for intrinsic part of the membrane. The first evidence for the attachment of ankyrin to the intrinsic domain via band 3 came from the observation that ankyrin and Band 3 are copurified (49) by Tritron X-100 It was found that 40% of the band 3 remain bound to skeletal proteins after extraction of all lipids. Other experiments demonstrated that 15% of Band 3 remained bound to the skeletal proteins. So we can draw the conclusion that about 15-40% of the band 3 remain bound to the skeleton. Other evidences suggests that ankyrin binding sites are present on the cytoplasmic domain of band 3 and all band 3 molecules have almost an equal affinity for ankyrin, and it was, therefore, suggested that band 3 existed as a tetramer in the membrane.

**4.1 Spectrin-actin Interactions:**

Protein 4.1 is associated with spectrin and actin in membrane

skeletons, and in vitro assays in many laboratories indicate that these three proteins participate in a ternary or higher order complex. Protein 4.1 does not interact directly with actin but does promote association of human erythrocyte spectrin with actin (50). Protein 4.1 interacts directly with the tails of spectrin tetramers, that have been localized by electron microscopy (28). Protein 4.1 associates with the isolated subunits of erythrocyte spectrin, although intact spectrin dimers are required to form a spectrin-actin-4.1 complex (51). Thus both spectrin subunits as well as 4.1 are involved in association with actin.

The stoichiometry of components in spectrin-actin-4.1 complexes depends on the relative concentration of each protein in the reaction. The ratio of spectrin dimer to 4.1 in 4.1-dependent actin complexes varied from 2:1 with low amounts of 4.1 to 1:2 at high concentrations of 4.1 (51). The implication of a 2:1 ratio of spectrin to 4.1 is that under these conditions each 4.1 is capable of promoting binding one and possibly two spectrin molecules to actin filaments.

**Association of Cytoskeleton with Bilayer:**

Several studies have suggested that cytoskeletal proteins interact with the phospholipids located in the cytoplasmic side of the membrane bilayer (Reviewed by Haest)(52). This has been speculated that the differential interactions between phospholipids and membrane proteins probably help in maintaining the asymmetric

distribution of phospholipids in red cell membrane. Spectrin, the major cytoskeletal protein of the red cell, has been speculated as the protein involved in the asymmetric distribution of phospholipids. This speculation is supported by the finding that covalent close linking of spectrin is invariably associated with loss of transmembrane phospholipid asymmetry (53, 54). The role of spectrin-membrane interactions in maintenance of the membrane phospholipid asymmetry has been supported by the observation in sickled cells (55). It has been shown that model membranes also (PS liposomes) bind significantly with the cytoskeletal extract (56, 57). Spectrin binding to PS liposomes is enhanced significantly in the presence of phosphatidylethanolamine (PE). PS liposomes have also been reported to interact with 4.1 polypeptide (58). The inference of all these studies is that the cytoskeletal proteins, spectrin in particular, contribute to the immobilization of the inner layer lipids, aminophospholipids in particular.

**Red Cell Membrane Lipids:**

Phospholipids and cholesterol are the two major lipids present in the erythrocyte membrane. Human red cell membrane contains four major [Phosphatidycholine (PC), Sphingomyelin (SM), phosphatidylethanol-amine (PE) and phosphatidic acid (PA)] types of phospholipids. The choline phospholipids are more predominant than the aminophospholipids, and are mainly localized in the outer monolayer (approximately 75% PC and 80% SM), whereas amino-

phospholipids are present (about 80% PE and 100% PS) mainly in the inner monolayer (59, 60). The fatty acyl chains of PE and PS are more unsaturated than that of PC and SM (61, 62), which appears to suitably account for the differences in the fluidities of the two monolayers (phase state asymmetry) (63).

The asymmetric distribution of phospholipids within the membrane is at least partly responsible for the membrane charge asymmetry, as only PS carries a net negative charge at the physiological pH. This phospholipid asymmetry in the erythrocyte membrane has physiological significance, since the presence of PS in the outer leaflet would tend to hyperactivate the blood coagulation system (64). Also it may be essential for maintaining an appropriate environment for optimal functioning of the membrane bound enzymes and other functional proteins (65).

Cholesterol is another major lipid constituent in the erythrocyte membrane and is present in the molar ratio (cholesterol: phospholipid) of 0.90. It regulates the permeability of biological membranes by affecting the membrane microviscosity.

Recently it has been shown that cholesterol can affect the turnover of polyphosphoinositides, which in turn would influence of the erythrocyte shape (66). In spite of an extensive work no definite conclusion has yet been drawn regarding the transbilayer distribution of cholesterol in the erythrocyte membrane, though there are evidences to suggest that the outer leaflet is enriched in cholesterol (67, 68, 69).

About 6-8% of the membrane carbohydrate is bound to lipids in the form of glycolipids which are present exclusively in the outer surface of the erythrocyte membrane (70).

## MALARIAL PARASITE INDUCED CHANGES IN RED CELL

### Receptors and Invasion Mechanism:

The life cycle of malarial parasite is marked by the periodic rupture of infected erythrocytes, release of infective merozoites and reinvasion, into erythrocytes (Fig. 4). The invasion process is a complex sequence of events and begins with the attachment of the merozoite to the erythrocyte. According to Miller (71) the total process of invasion takes about 30 seconds and consists of four phases. Initially, the parasite recognises and attaches to the red cell, which is followed by deformation of the target cell membrane. Subsequently, the merozoite enters the erythrocyte by way of invagination of the red cell membrane and finally after the entry is completed, the membrane seals of (Fig. 5). This invasion process has also been described in detail by light and electron-microscopic techniques (72). The binding of the invading merozoite to the red cell is highly specific and is mediated by specific cell surface receptors (73). McGhee reported that P.lophurae merozoites have much higher affinity for duck erythrocytes than chicken erythrocytes. In 1973, Miller et al (74) observed that enzyme treated red cells show resistance to P.knowlesi and P.falciparum infection. This confirmed parasite specific receptors

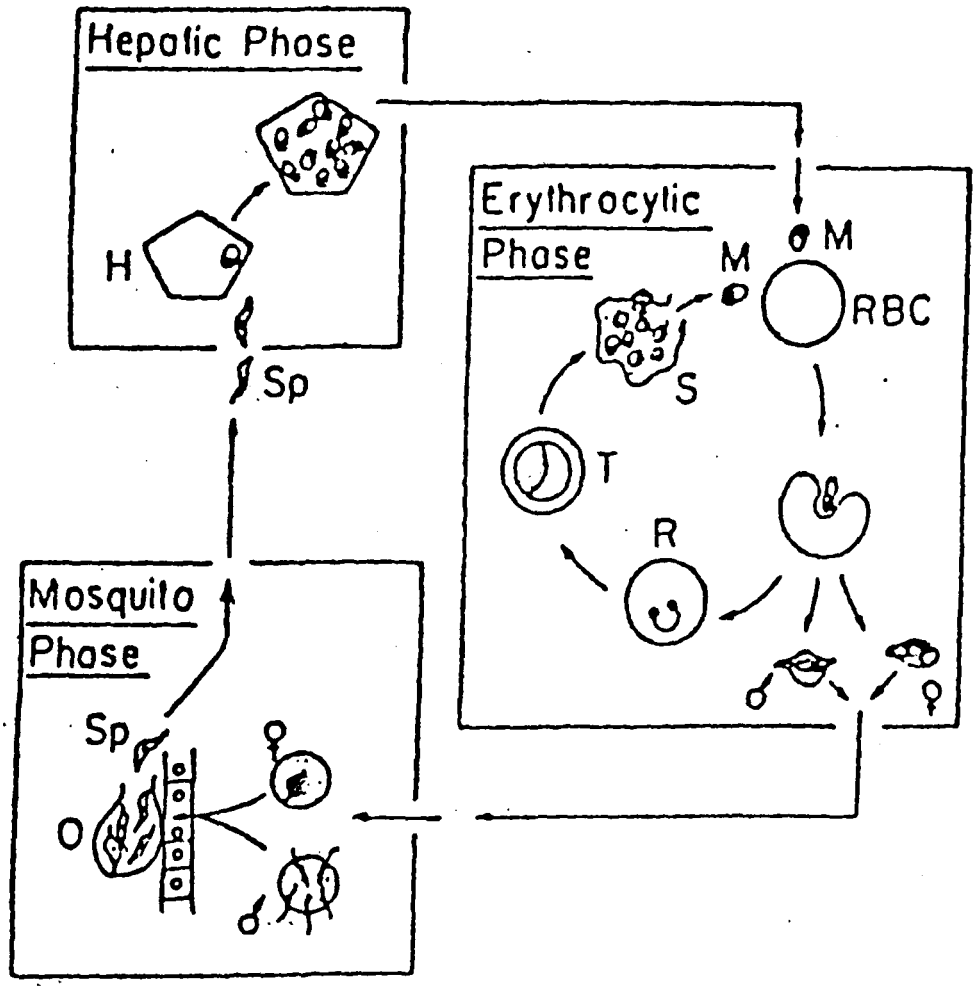



**Fig. 4:** The life cycle of plasmodium. A schematic representation of various phases in the life cycle of the species P.falciparum. Not drawn to scale, M, Merozoite; RBC, red blood cell; R, ring; T, trophozoite; S, schizont; O and O, male and female gametocyte; O, oocyte; Sp, sporozoite; H, Hepatocyte. From Breuer, W.V. (115).

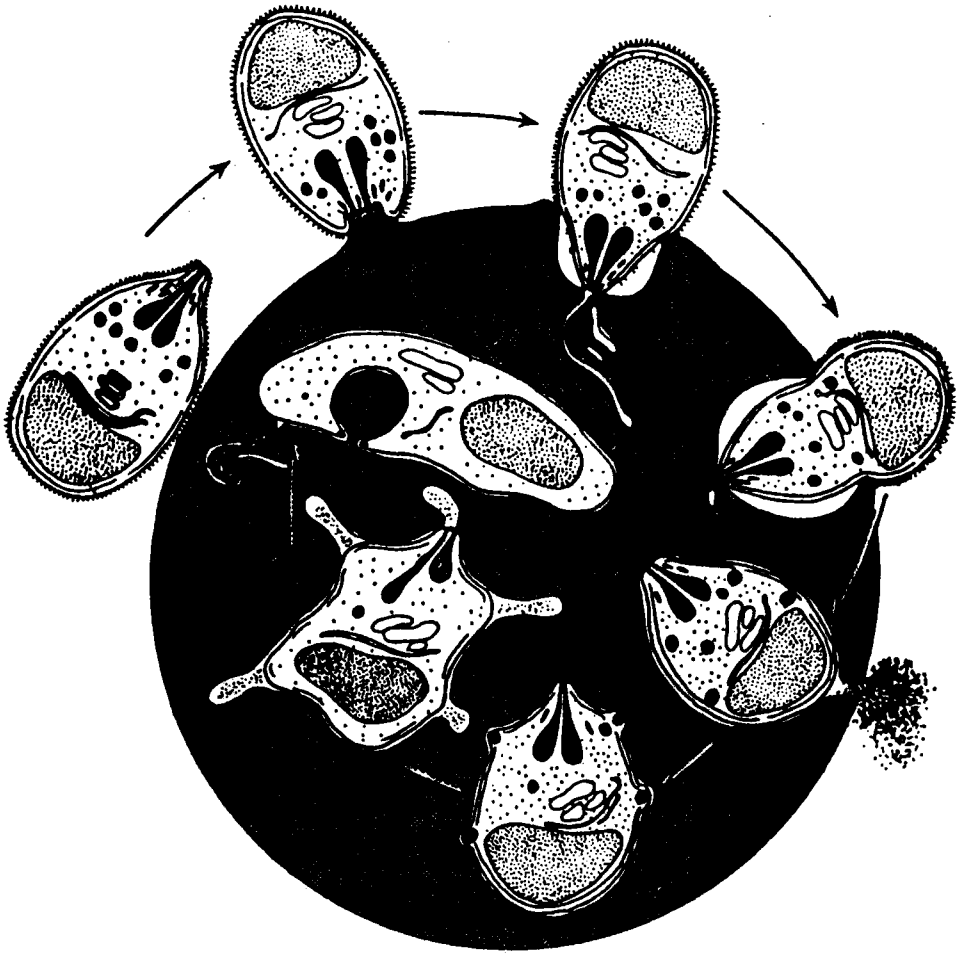


**Fig. 5:** Major stages in the invasion of a malarial merozoite into an erythrocyte.

on red cell. The work of Miller et al (75) revealed that human erythrocytes lacking the duffy blood group are resistant to P.vivax invasion. Also, P.vivax invasion of duffy positive human red cell is blocked by antiduffy antibodies.

However, there are two objections to these observations. First duffy negative red cells become susceptible to P.knowlesi invasion on treatment with neuraminidase or trypsin and yet remain duffy negative. Secondly, P.knowlesi attaches to duffy negative human erythrocytes but fails to enter them. Thus it may be considered that it is the interiorization rather than recognition that is defective in duffy negative erythrocytes (76).

Glycophorin A as well as glycophorin B and C are reported to be the possible receptors for the malarial merozoite with the observation that En($a^-$) erythrocytes are poorly invaded by P.falciparum. This was also confirmed with the finding that anti-glycophorin A as well as glycophorin inhibit invasion of P.falciparum in vitro (77). Removal of sialic acid moieties and the N-terminus of glycophorin C from red cell by enzymatic treatment also affects the invasion process. From this observation it has been suggested that sugar residues of glycophorin may be involved in the binding of the merozoite to the erythrocyte and corresponding lectin like polypeptides have been identified on the surface of P.falciparum merozoites. All these evidences support the involvement of glycophorin in the merozoite association with the red cells. Despite all these evidences the involvement of glycophorins as the receptor

for P.falciparum has recently been questioned. Okoye and Bennett (78) have reported the involvement of band 3 as a possible receptor for the P.falciparum merozoite. This hypothesis shows that band 3 protein (1 million copies per red cell) participates in malarial invasion in a highly specific manner. It may thus be concluded that the erythrocyte receptor for the malarial parasite is not yet fully characterized, but glycophorin A and band 3 do help the parasite's entry into the red cell. After initial attachment of the merozoite to the red cell, the merozoite reorients itself such that the apical end of the parasite is opposed to the erythrocyte membrane. This is followed by formation of a junction between the apical end of the merozoite and the erythrocyte membrane (79). Studies by Aikawa et al (80) have confirmed that IMP (Intramembranes particles) which represents integral membrane proteins, band 3 and glycophorin, rearrange themselves at the site of plasmodium entry, just as in endocytosis or membrane fusion.

Another important feature of the invasion is that any alterations in the cytoskeleton-transmembrane protein interactions should inhibit invasion. This is supported by the observations that P.falciparum fails to invade ghosts having cross-linked spectrin and reduced invasion in cells having abnormal spectrin (81). ATP depletion leads to aggregation of IMP, which in turn may suitably account for reduced invasion of red cells having decreased ATP levels (82). P.knowlesi invasion to monkey red cell is inhibited by modifying cytoskeleton with Colchicine and Vinblastin which act as crosslinker for cytoskeleton.

Other Changes in Host Cell Membrane:

Drastic morphological changes have been observed in the discositic shape of red cell with the maturation of the parasite. This includes surface indentations, capping of erythrocytes and variable and irregular surface protrusions. In case of P.falciparum asexual parasites induce the formation of knobs and underlying electron dense material (EDM) at the erythrocyte membrane (83). These knobs express new surface antigens. Recent freeze-fracture electron microscopic studies have visualized knobs as conoid projection of the protoplasmic fracture face with the depression of the exoplasmic fracture face (EF) of the erythrocyte membrane (84). These knobs form focal junctions with the endothelial cell membranes or with the knobs of other erythrocytes, resulting in the sequestrations of the infected erythrocytes of certain species along the vascular endothelium (85). The sequestration of these cells in deep tissues may be favourable for the differentiation and development of the parasite.

Small surface invagination called caveolae, are observed in erythrocytes infected with P.knowlesi, P.ovale or P.vivax. These invaginations are surrounded by small vesicles and caveolae-reside complex correspond to Schuffner's dots observed under light microscopy. There is decrease in the normal density of IMP in malaria infected red cells. This reduction in IMP was much more marked in areas where schizont and host cell plasma membranes were in close apposition and has been thought to result

in expansion of the host cell membrane, and also in an increase in lateral movement of transmembrane proteins. Furthermore, the alteration in IMP density depends on the type of erythrocytes. There was no change in IMP distribution in P.falciparum infected human erythrocytes whereas aotus erythrocytes infected with P.falciparum showed an aggregation of IMP over the P-face of the knobs. This variation has been correlated to the differences in cytoskeleton-band 3 interactions in the two types of red cells (86). However, Allred et al (1984) observed series of changes associated with PF leaflet of human erythrocytes infected with P.falciparum. These alterations include: (a) IMP clustering in the central core of the knobs surrounded by an IMP-freeze zone and concentric IMP ring (b) erythrocyte membrane deformation concomitant with a loss of IMP organization and (c) parasite development did not affect IMP densities in the PF but a decrease was noted in EF of schizont infected erythrocytes.

Membrane Lipids of Infected Red Cells:

Malarial parasite infected red cells have altered membrane lipid composition and fluidity. The cholesterol level in red cells have been found to be reduced (87). Alterations in membrane phospholipid organization have been reported in erythrocytes harbouring different developmental stages of parasites. The first evidence of alteration of membrane phospholipid asymmetry in malarial parasite infected red cell was reported by Cooper and Miller (88). It was supported by Gupta and Misra (89)who reported

alteration in the distribution of aminophospholipids in P.knowlesi infected red cells using enzymatic and chemical probes. It has been observed (90) that the increase in aminophospholipids (PS & PE) in the outer leaflet of the membrane is stage dependent or much more pronounced in the late-stage of the parasite development.

The proportions of fatty acids are also altered markedly as a result of parasitization, like a decrease in polyunsaturated fatty acids and an increase in saturated octadecanoic acids have been reported in these cells.

These changes in membrane lipids may suitably account for altered fluidity of plasma membrane, which has been observed in P.berghei and P.falciparum infection with fluorescence and electron spin resonance spectroscopies(114). The changes in membrane fluidity is also stage dependent (90, 91).

Changes in Host Cell Carbohydrate Organization:

Membrane protein bound carbohydrates are also altered in parasitized red cells. Parasitized red cells show enhanced binding of Concanavalin A and WGA as compared to normal mouse red cells. The sialic acid content in schizont infected red cell is found increased as compared to uninfected red cells (92).

Changes in Host Cell Membrane Proteins:

Malarial parasite introduces two types of changes in red cell membrane proteins. First, it modifies the red cell membrane

Table 1: Literature data concerning the disposition of Plasmodium proteins in plasma membranes of parasitized erythrocytes.

| *Plasmodium* species | Parasite proteins ($kD_a$) | Membrane disposition | Method used |
|---|---|---|---|
| *P. chabaudi* | 105 | cryptic | IFM |
| | 93 | cryptic | IFM |
| | 76 | cryptic | IFM |
| *P. berghei* | 120 | cryptic | IFM |
| | 65 | cryptic | IFM |
| | 60 | outside | RI |
| | 54 | outside | RI |
| | 46 | cryptic | IFM |
| | 40 | outside | RI |
| | 31 | cryptic | IFM |
| | 13 | cryptic | IFM |
| *P. vinckei* | 160 | cryptic | IFM |
| *P. knowlesi* | 230 | cryptic | RI |
| | 200 | outside | RI |
| | 140 | outside | RI |
| | 130 | outside | RI |
| | 125 | outside | RI |
| | 102 | outside | RI |
| | 90 | outside | RI |
| | 74 | outside | RI |
| | 42 | outside | RI |
| *P. falciparum* | 300 | cryptic | IEM |
| | 290 | outside | RI |
| | 250 | outside | IEM |
| | 155 | outside | IFM |
| | 105–85 | cryptic | IEM |
| | 95–80 | cryptic | IEM |
| | 90 | outside | RI |
| | 65 | outside | RI |
| | 55 | outside | RI |
| | 45 | outside | RI |
| | 35 | outside | RI |
| | 20 | outside | RI |

'Out side', exposure of parasite proteins on the surface of infected erythrocytes; 'cryptic', association of parasite protein with the cytoplasmic face of the host cell plasma membrane or not accessible from the outside in intact erythrocyte.

proteins and secondly it introduces some new proteins in the red cell membrane to facilitate its entry and subsequent growth in host cell (Reviewed by Gupta, 93). The modifications in the host cell membrane proteins include the degradation of certain membrane proteins notably spectrin, band 4.1 polypeptide, glycophorins and components of Band 5. The degradation of these proteins are thought to be the new bands that appear in the electrophoretograms of malarial parasite infected red cell membranes. However, this is not yet known as to how these changes in the membrane proteins are brought about by the intracellular parasite. But it has been speculated that it might be because of the activation of $Ca^{++}$ induced proteases (94).

Some new proteins have been observed in erythrocyte membranes harbouring different developmental stages and species of malarial parasites. By metabolic surface labelling and also by immuno-precipitation techniques it has been shown that a large number of these new polypeptides are of parasite origin. Literature data concerning the disposition of plasmodium protein in plasma membranes of host cell is shown in Table 1. The new proteins that appear in the host cell plasma membrane are thought to carry out two functions. Firstly, they may contribute to the metabolic requirement of the red cell and secondly, they may help to protect parasitized cells against the host defense system. A protein of 122 kD was demonstrated on the surface of P.knowlesi-infected erythrocytes by pyridoxal phosphate/sodium borohydride catalyzed

labelling. This protein is thought of to be an additional anion transporting system, synthesized and inserted into the host cell membrane by the parasite (95).

Abnormal red cells are eliminated from the system by the spleen. Because the parasitized red cells have deformed morphology and as such should be eliminated by the spleen. The presence of knob like structures on the surface of erythrocytes infected with certain species of malarial parasite help these cells to escape destruction by spleen by sequestring them in deep endothelial tissues. The knobby (K+) P.falciparum infected erythrocyte adhere to the endothelial cells because of cytoadherence property of the knobs (96). Another mechanism of escape is the expression by P.knowlesi schizont-infected erythrocytes of two types of surface antigenic proteins. One group consists of constant antigens and the other highly variable antigens referred to as variant antigens. The expression of variant antigens by the parasite may divert the immune response away from the constant antigens and thus could help in escape of infected erythrocytes from destruction by the spleen (97, 95). Apart from the appearance of neo proteins there are reports that the permeability of the infected red cells is changed. Pore like structures have been observed, which may account the new permeability pathways observed in the cells (98). The actual number of pores increases with the maturation of the parasite (98). In parasitized cells the activity of $Ca^{++}$ and $Mg^{++}$ ATPase is significantly altered and accumulation of $Na^{+}$ inside the

infected red cells is observed due to the failure of $Na^+/K^+$ ATPase (99).

## AIMS AND OBJECTIVES OF THE STUDY

In order to invade the red cells, the malarial parasite brings about drastic alterations in the membrane lipid organization. The parasite also alters the cytoskeleton at the site of its entry. It has been suggested that the major cyoskeleton protein, spectrin gets degraded on parasitization (100, 101, 92, 107). However, it is not yet clear whether this degradation is via activation of the erythrocyte membrane bound proteases or caused by parasite proteases. Degradation or redistribution of spectrin in host cell should affect the organization of the cytoskeleton and in turn may affect the cytoskeleton phospholipid interaction. Also, the insertion of new proteins in the host cell membrane may lead to structural alteration at the insertion site. This could lead to alterations of the interaction between the lipid bilayer and cytoskeleton. In order to understand the modification brought about by the parasite in the organization of the cytoskeleton at the molecular level, the present study was undertaken in P.knowlesi-infected monkey red cells.

The objectives of the study are defined as follows:

i) The difference in protein pattern of the host cell membrane (Normal and parasitized in different stages of infection) by SDS-PAGE.

ii) The protein pattern of different glycoproteins in the host cell membrane in schizont stage.

iii) Characterization/Confirmation of the new proteins as possible degradation products of spectrin by crossed-immunoelectrophoresis.

iv) Extractability of cytoskeletal-proteins from host cell membrane.

v) Spectrin organization in low ionic strength extract by gel filtration chromatography.

# MATERIALS AND METHODS

## MATERIALS

Healthy rhesus monkeys (male), weighing 5-6 kg were procured from the CDRI primate house. Affi-Gel 731 was obtained from Bio-Rad laboratories. Ficoll 400 and Conray-420 were purchased from Pharmacia-Fine Chemicals and May & Baker Ltd., respectively. Phenylmethylsulfonyl-fluoride (PMSF), Pepstatin A, Leupeptin, Methylene bis-acrylamide were obtained from Sigma Chemical Co. (USA). Ammonium persulfate and N, N, N', N'-tetramethylethylene diamine were purchased from May and Baker and BDH, respectively. All other chemicals were obtained from Qualigens or SISCO research laboratories (India). Acrylamide was recrystallized before use.

## METHODS

### P.knowlesi Infection in Rhesus Monkeys:

Healthy rhesus monkeys (Macaca mulatta), weighing 4-6 kg, were infected with Plasmodium knowlesi, and kept in light between 7 hrs and 19 hrs to maintain synchronicity of the infection. All other conditions were the same as described by Banyal et al (105).

The W1 strain of P.knowlesi was used. Infection of each animal was initiated either by an intravenous injection of 0.75-1.5 ml of buffered cell suspension that had been frozen in liquid nitrogen. The frozen cell stocks of infected cells were prepared by mixing two parts infected blood (preferably ring stage, anti-

coagulated with heparin 20 U/ml) with one part of 3:7 glycerin/ 5 mM Na phosphate, 150 mM NaCl, pH 7.4 (v/v). The mixtures were cooled and then frozen at -70°C in liquid nitrogen.

**Evaluation of Parasitaemia:**

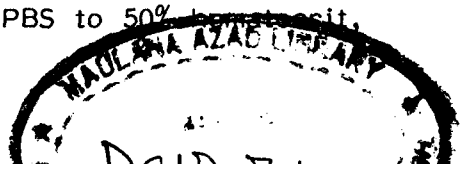
The progress of parasite development in infected animals was monitored by quatidian blood smears. For this, standard uniform blood smears on the glass slides were stained with Geimsa. The extent of infection was ascertained by counting the proportion of the infected cells.

**Fractionation of Infected Erythrocytes:**

Parasitized red cells were separated from the non-parasitized red cells and leucocytes by Ficoll-Conray density gradient. The Ficoll-Conray mixture was prepared essentially as described by Singhal *et al.* (106). A stock Ficoll 400 solution of 9% (w/v) was made in normal saline and diluted to appropriate densities by addition of 33% Conray 420 (v/v) prepared in distilled water.

**Isolation of Parasitized Erythrocytes:**

*P. knowlesi* infected monkeys were bled at high parasitaemia in heparinized PBS (5 mM sodium phosphate, 150 mM NaCl pH 7.4) and centrifuged at 1,075*g* for 4 min. The plasma was carefully aspirated and the top darkbrown layer of infected red cells was taken out carefully. The cells were washed three times with PBS. The washed cells were suspended in PBS to 50% hemat[illegible]



and the cell suspension was loaded on a mixture of Ficoll-Conray in a ratio of 1:2 respectively. The cells were centrifuged at 700 g for five min. The infected red cells form a clear band at the Ficoll-Conary/buffer interphase. These cells were washed thrice with PBS. The leucocyte contamination in these parasitized red cell was removed by layering them (50% hematocrit) on Ficoll-Conray (density 1.080) and spinning at 700 g for five min. In this case, equal volumes of the gradient and diluted red cells were used. The infected cells thus obtained had 2-10% contamination of the nonparasitized erythrocytes. Leucocyte contamination was less than 0.1%. The trophozoite and schizont infected erythrocytes were purified by using a density of 1.076 and 1.080 of Ficoll-Conray respectively. The parasitized red cells so obtained were found intact, as judged by light microscopy.

**Preparation of Host Cell Membrane:**

The parasite-free host erythrocyte membrane was prepared as follows: the cells were lysed with 20 mM sodium phosphate pH (7.5), containing 0.1 mM EDTA, 0.2 mM PMSF and 20 ug/ml each of Pepstatin A and Leupeptin. The lysate was immediately centrifuged at 700 g for 2 min at 2-4$^{o}$C. Extreme precaution was taken to handle this lysed solution. The supernatant was carefully aspirated leaving behind a pellet consisting of intact parasites and few unlysed cells. The supernatant was centrifuged three times in the same way and the pellet was discared each time.

The membranes were recovered by spinning the final supernatant at 30,000 g for 20 min at 4°C and washed two times with 5 mM sodium phosphate containing 0.1 mM EDTA pH (7.4) prior to further use. The purity of the membrane was checked by light-microscopy and also by assaying the parasite-specific enzyme glutamate dehydrogenase.

The normal monkey erythrocyte ghosts were prepared by the method of Fairbanks et al (36).

**Extraction and Purification of Spectrin:**

Spectrin was extracted from the normal and parasitized monkey red cell membranes with low ionic strength buffer (0.3 mM sodium phosphate, 0.1 mM EDTA, pH 8.0) containing PMSF (0.2 mM), Leupeptin and Pepstatin A (20 ug each/ml). After washing membrane pellet with the buffer spectrin extraction was performed by incubating the membranes with 5-7 volumes of the extraction buffer either 36 hr at 4°C or for 30 min at 37°C. After completing the incubation, the suspension was centrifuged at 100,000g for 60 min in Sorvall AH-627 rotor and the supernatant, which contained spectrin, was carefully taken out.

Purification of spectrin and tetramer-dimer separation were carried out according to the procedure of Gratzer et al (13). Briefly, the crude spectrin, containing free spectrin and actin as well as spectrin-actin-4.1 oligomers and traces of haemoglobin, was concentrated to a protein concentration of 1 mg/ml by using

Amicon-50 ultrafiltration cones. This was applied to a (2.5x55 cm) or (1x40 cm) column of Sepharose CL-4B, equiliberated with 20 mM sodium phosphate, 0.1 M NaCl, 2 mM $NaN_3$, pH 7.6. The column was eluted at about 18 ml/hr and 2 ml or 2.5 ml fractions were collected. Protein in the effluent was monitored by absorbance at 280 nm and analysed for purity by SDS- polyacrylamide gel electrophoresis (36, 103).

**Gel Electrophoresis:**

Tris-glycine system of Laemmli (103) was used for SDS-polyacrylamide gel electrophoresis. Routinely a separating gel of 10% acrylamide (pH 8.8) and a stacking gel of 5% acrylamide (pH 6.8)were used.

Stock solutions of 30% acrylamide containing 0.8% bis-acrylamide, 1M Tris (pH 8.8 and 6.8) and 20% SDS were prepared and used whenever required. Protein samples were prepared to give a final concentration of 2% (w/v) SDS, 0.5% (v/v) 2-mercaptoethanol, 0.0625 M Tris HCl, pH 6.8 and 10% (v/v) glycerol with a trace of bromophenol blue as a tracking dye. Samples were then heated in a boiling water bath for about 3 min. The electrode buffer contained 0.025 M Tris, 0.2 M glycine and 0.2% SDS. The protein bands were detected by staining the gels with Coomasie-Brilliant Blue R-250. In some experiments continuous gel electrophoresis system of Fairbanks et al (36) was used for protein analysis.

**Antispectrin Antiserum:**

Antiserum against purified spectrin was raised in healthy

rabbits by injecting the protein, emulsified in Freund's complete adjuvant, subcutaneously at multiple sites (60-70 sites). After 65 days, booster doses were given in Freund's incomplete adjuvant.

**Crossed Immunoelectrophoresis:**

The crossed immunoelectrophoresis was carried out using the known procedure (102). Briefly, the host cell membranes were subjected to SDS-PAGE in 1.5 mm thick slab gels using the procedure of Laemmli (103). The runs were terminated when the tracking dye migrated to a distance of 10-12 cm. The gel strips containing ghost proteins were trimmed and washed for 30 min with a buffer containing 38 mM Tris, 100 mM glycine (pH 8.7) and 1% Triton X-100 A 1% agarose gel (15x8 cm) was prepared in 38 mM Tris, 100 mM glycine (pH 8.7), and 3.5% Triton X-100. At cathodic end of the agarose gel, a well was cut of the size of polyacrylamide gel strip. The polyacrylamide gel strip was then transferred to this well and the gaps were sealed by application of a few drops of warm agarose (with 3.5% TritonX-100). Simultaneously, a well was prepared (size 13x3 cm) in the agarose gel at the anodic side for layering antibody containing agarose. Agarose solution (1%), containing, 38 mM Tris, 100 mM glycine, 2% TritonX-100 and 100 ul of antispectrin serum per ml of agarose solution, was poured in the preformed well. Electrophoresis was carrid out 2V/cm for about 16 hr at room temperature, using Tris-glycine buffer (pH 8.7), without TritonX-100. The polyacrylamide gel strip was removed prior to washing and staining of the agarose gel.

**Periodic Acid Schiffs Staining of Gels:**

The carbohydrate specific staining of polyacrylamide gels was performed essentially according to Fairbanks et al (36). Because, a high concentration of SDS produced an intesne background, the SDS was removed before PAS staining by suspending the gel in the following solutions at the room temperature for the stated times; no less than 50 ml/gel was used at each stage: (1) 25% isopropyl alcohol, 10% acetic acid; overnight; (2) 10% isopropyl alcohol, 10% acetic acid; 6-9 hr; (3) 10% acetic acid; overnight.

After this treatment the gels were treated with staining reagent with gentle shaking at room temperature in the following sequence: (1) 0.5% periodic acid; 2 hr; (2) 0.5% sodium arsenite, 5% acetic acid; 30-60 min; (3) 0.1% sodium arsenite, 5% acetic acid; 20 min repeated twice; (4) 5% acetic acid, 10-20 min and repeated twice. The gels were then soaked in Schiff's reagent overnight. Rose pink bands appeared after 5-10 min in the Schiff's reagent and intensified as the reagent penetrated to the centre of the gels. The unreacted Schiff's reagent was removed by soaking the gel strips in 0.1% sodium metabisulphite in 0.01 N HCl with intermittant changes.

**Glutamate Dehydrogenase Assay:**

The lytic effect of 20 mM sodium phosphate (pH 7.4) on the parasite was determined by lysing the infected erythrocytes and pelleting the parasite at 750 g. The pellet was immediately

washed with PBS (5 mM sodium phosphate, 155 mM sodium chloride pH 7.4) three times. An aliquot of this pellet was sonicated in 20 mM sodium phosphate buffer (pH 7.4) in ice cold condition and suspension centrifuged at 20,000 g (30 min). This supernatant was used for standard assay of glutamate dehydrogenase activity. Parasite contamination in the membrane preparation was checked by taking 200 ug of host cell membrane protein and 200 ul of parasite lysate (obtained after sonication) in separate test tubes. To these tubes, 0.2 ml of ammonium acetate (1 M stock) and 0.10 ml of NADH (2 mM stock) was added. The volume of the assay system was then made to 1.7 ml with 20 mM sodium phosphate (pH 8.0). After measuring the preliminary non-specific reaction at 340 nm, 0.30 ml of the substrate, ∝ ketoglutarate (100 mM stock/pH 8.0) was added and change in optical density at 340 nm was recorded. Conversion of NADH to NAD at 340 nm was used was the criteria of enzyme activity (104).

# RESULTS

**Assessment of Membrane Purity:**

Since the success of the proposed study largely depended on the purity of the erythrocyte membranes, the isolated membranes were examined for their purity. The methods currently available to assess the purity of erythrocyte membranes include microscopic observation for the presence of intact parasite or its organelles, and determination of the parasite-specific enzymes in the erythrocyte membrane preparations. These methods though reliable, but fail to detect the contamination of the parasitophorous vacuole membrane which originates from the erythrocyte plasma membrane at the time of merozoite entry into the red cell and encircles the intracellular parasite. There are no accepted ultrastructural immunochemical or biochemical markers for this membrane. Therefore, the presence of this membrane in the erythrocyte membrane preparation is difficult to ascertain.

The membranes recovered from P.knowlesi-infected erythrocytes after differential centrifugation were virtually free of parasites as judged by light microscopy and on the basis of parasite's soluble enzyme marker, glutamate dehydrogenase. No enzyme activity was detected in any of the membrane preparation.

**Comparison of Monkey and Human Red Cell Membrane Proteins:**

Analysis of Coomassie blue-stained SDS-PAGE gels have revealed, that there was no significant difference in both electrophoretograms. The comparative study of the both electrophoreto-

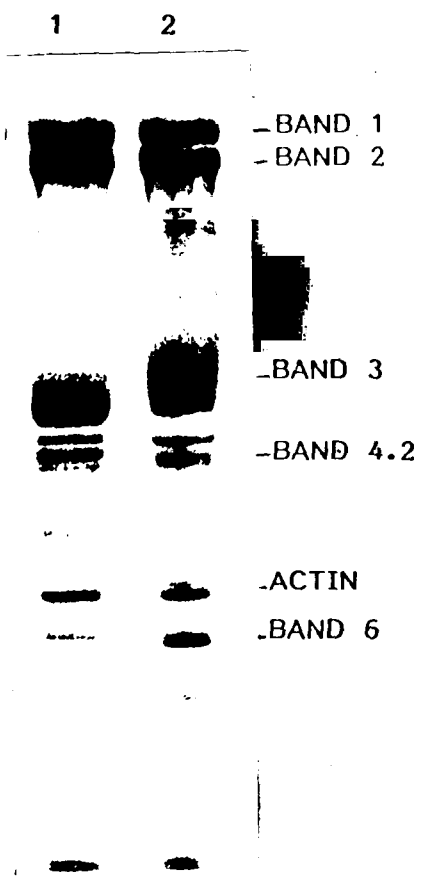



**Fig. 6:** SDS-polyacrylamide gel electrophoresis of erythrocyte membrane proteins from: Lane 1: normal monkey red cells, Lane 2: normal human red cells.

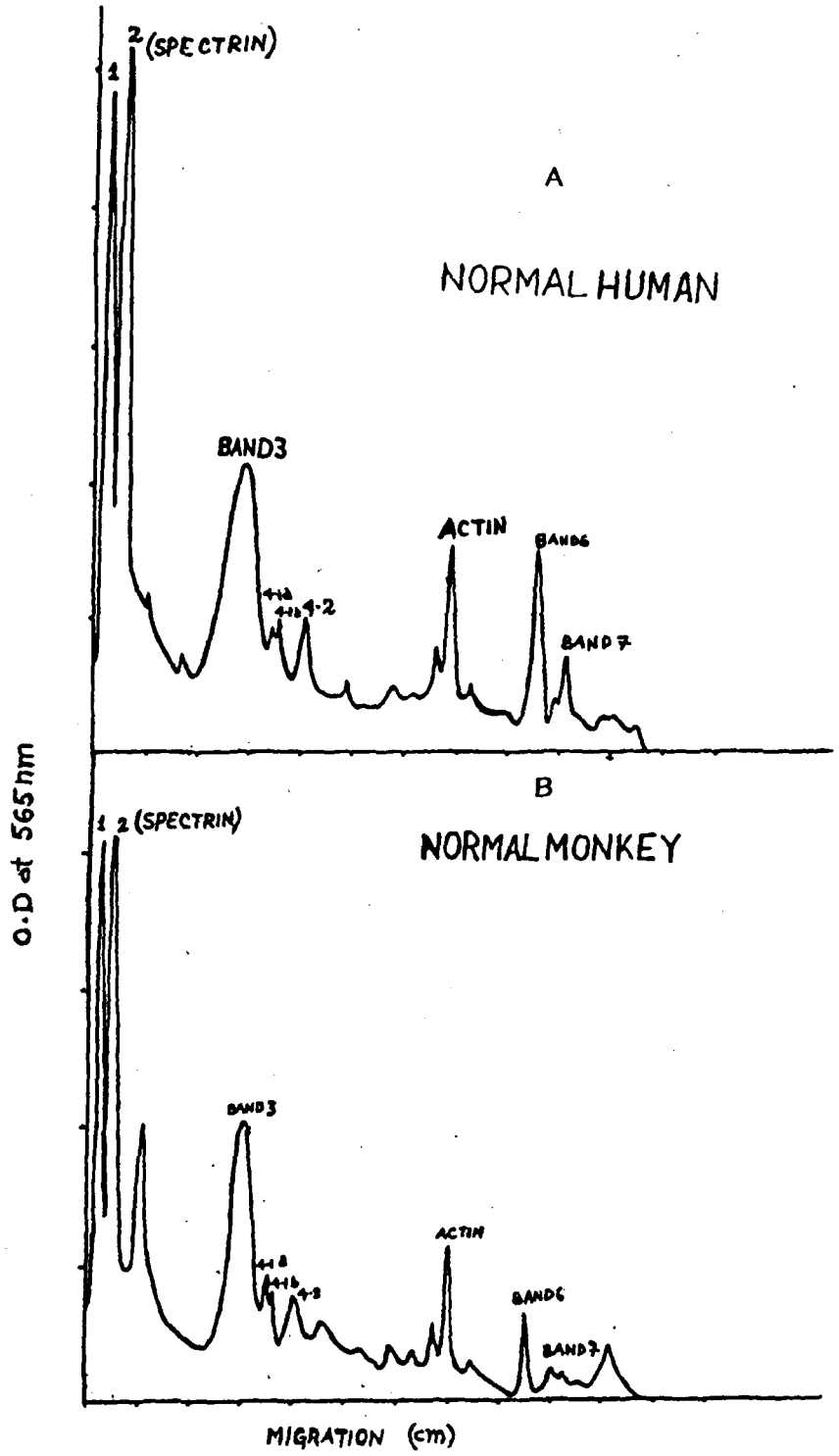



**Fig. 7:** Densitometric scans of Coomassie blue stained SDS-PAGE gels of erythrocyte membrane prepared from:
A - Normal human erythrocytes
B - Normal monkey erythrocytes

Table 2

Composition of Major Membrane Proteins of Normal and P.knowlesi-infected Rhesus Monkey Erythrocytes

| Percentage of Coomassie Blue Stained (Concentration) | | | | | | | |
|---|---|---|---|---|---|---|---|
| Band No. | Normal Human Erythrocytes | Band No. | Normal Rhesus Monkey Erythrocytes | Band No. | Trophozoite infected erythrocytes | Band No. | Schizont Infected Erythrocytes |
| 1 | 19.313 | 1 | 11.025 | 1 | 11.25 | 1 | 14.979 |
| 2 | 21.969 | 2 | 20.089 | 2 | 18.237 | 2 | 19.552 |
| | 0.615 | | | | | $S_1$ | 0.483 |
| 2.1(Series) | | 2.1 series | 2.658 | 2.1 (Series) | 4.380 | 2.1 (Series) | 3.403 |
| | | " | 0.228 | $T_1$ | 0.167 | $S_2$ | 1.114 |
| | | " | 0.136 | $T_2$ | 2.084 | $S_3$ | 0.453 |
| | | " | 0.126 | | 0.102 | | |
| | | | | $T_3$ | 0.707 | | |
| 3 | 34.012 | 3 | 34.167 | 3 | 24.496 | 3 | 22.361 |
| 4.1 a | 0.313 | 4.1 a+b | 2.293 | 4.1 a+b | 2.215 | 4.1 a+b | 2.262 |
| 4.1 b | 0.525 | | | | | | |
| 4.2 | 3.909 | 4.2 | 4.594 | 4.2 | 2.571 | 4.2 | 2.746 |
| | | | | | | $S_4$ | 1.240 |
| 4.5 (Series) | 0.530 | 4.5 (Series) | 0.254 | $T_4$ | 0.610 | $S_5$ | 1.103 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.953 | | 0.104 | | | 4.5 (Series) | 3.896 |
| 0.253 | | 1 | 4.9 | 0.730 | | 2.235 |
| | | | | | | 2.450 |
| 0.764 | 4.9 | 1.845 | | | 4.9 | 1.186 |
| 6.135 | 5 | 9.294 | 5 | 4.440 | 5 | 9.378 |
| 0.829 | | | | | | |
| 7.485 | 6 | 3.066 | 6 | 1.378 | 6 | 1.233 |
| | | | | 3.591 | $S_3$ | 0.924 |
| 1.725 | 7 | 0.921 | 7 | 5.605 | | 2.585 |
| | 8 | 2.631 | 8 | 1.162 | | 0.407 |

grams are given in Table 2. Densitometry scans of these gels, shown in Fig. 6, indicate that the relative concentration of spectrin in the human red cells membranes is higher than in the monkey red cell membranes. Also, the amount of band 6 protein appeared higher in the human cells as compared to monkey erythrocytes. The relative concentrations of other proteins were almost similar in both the cases (Fig. 7).

**Membrane Proteins of Infected Erythrocytes:**

No significant changes seemed to occur in the membrane proteins of the host monkey erythrocytes at any stage of P.knowlesi infection, viz. trophozoite and schizont (Fig. 8,9). The relative concentrations of the major proteins like spectrin (Band 1 and 2) and Band 3 protein in the parasitized, cells were similar to that in the uninfected normal erythrocytes. Densitometric scans of these gels are given in Fig. 10 and 11. Also the intensities of bands 4.1 and 4.2 were not significantly altered (Table 2). However, in the membranes of the parasitized erythrocytes, there were some new proteins which were absent in the normal erythrocyte membrane. The apparent molecular weights of these new proteins, as determined by SDS-PAGE, were about 163 kD, 107 kD, 90 kD, 70 kD, 55 kD and 23 kD (Fig. 8,9 ). Besides these new proteins, the intensity of the erythrocytes membrane protein corresponding to band 6 was significantly reduced after infecting the cells with P.knowlesi (Fig. 8,9 ). Also, the concentration of the band 7 protein appeared to increase in the infected cells, which was consistant with the earlier report (107) (Fig. 8,9).

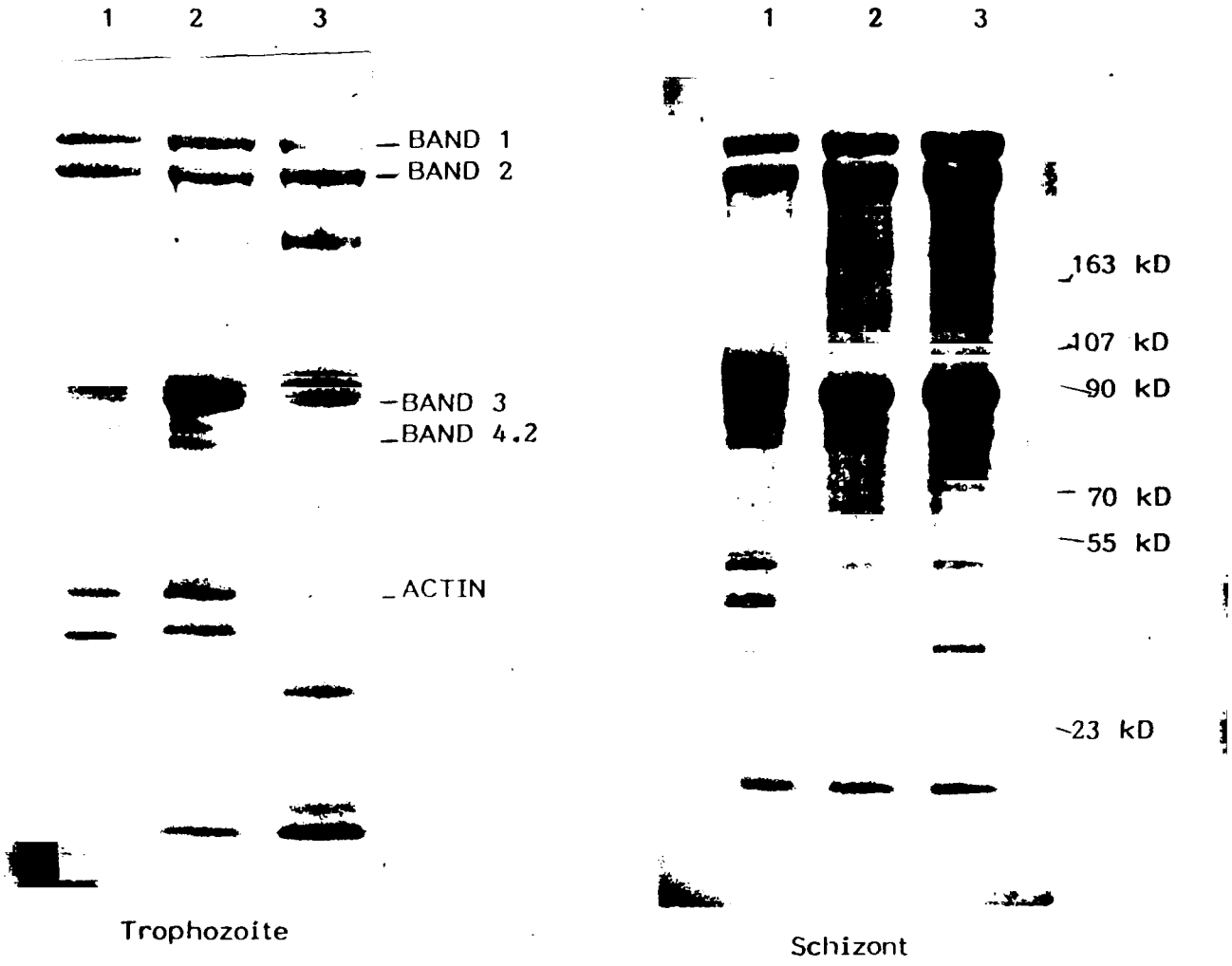



**Fig. 8:** SDS-polyacrylamide gel electrophoresis of erythrocyte membrane proteins from:

a) - Normal human red cell
b) - Normal rhesus monkey red cell
c) - Trophozoite - infected rhesus monkey red cell

**Fig. 9:** SDS-polyacrylamide gel electrophoresis of erythrocyte membrane proteins from:

a) - Normal human red cell
b) - Normal rhesus monkey red cell
c) - Schizont-infected rhesus monkey red cell

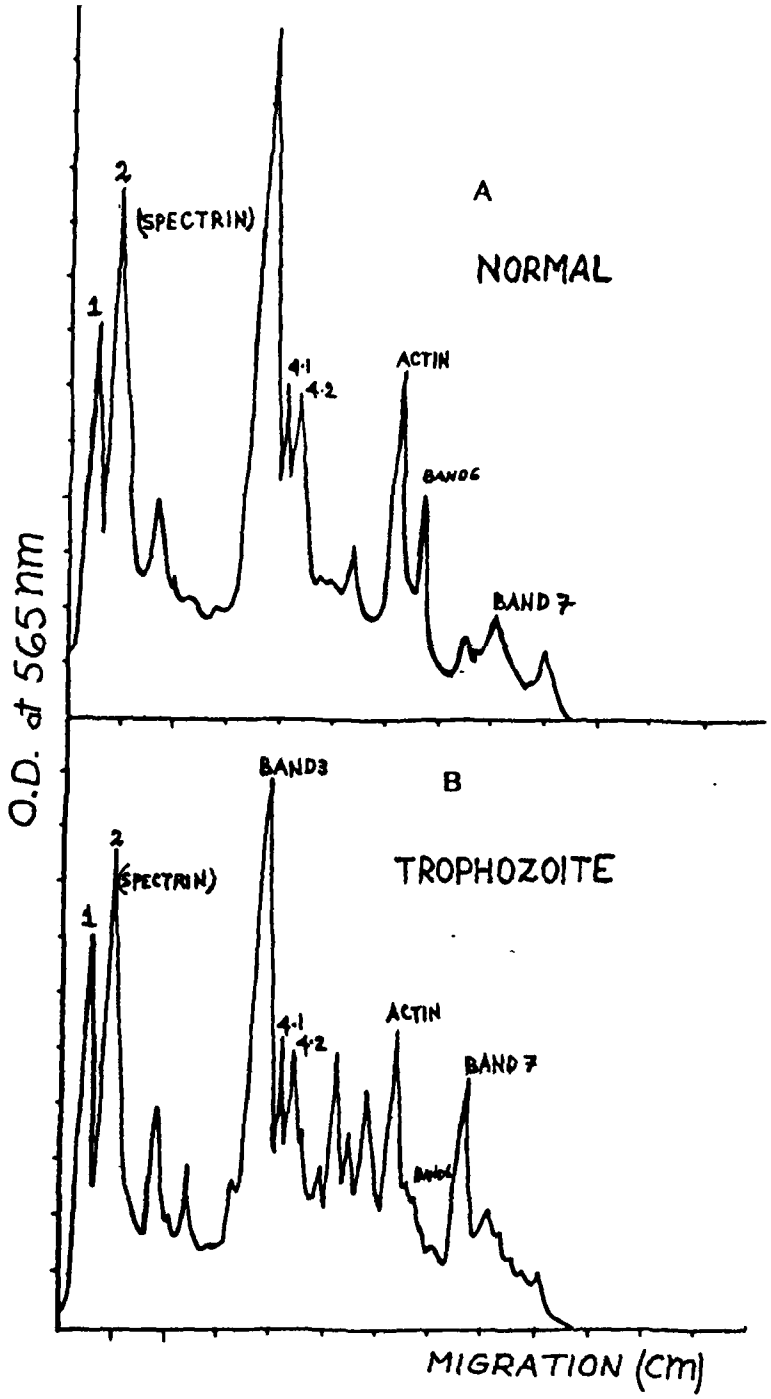



**Fig. 10:** *Densitometric* scans of Coomassie blue stained SDS-PAGE gels of erythrocyte membrane prepared from:
A - Normal monkey erythrocyte
B - Trophozoite-infected monkey erythrocyte

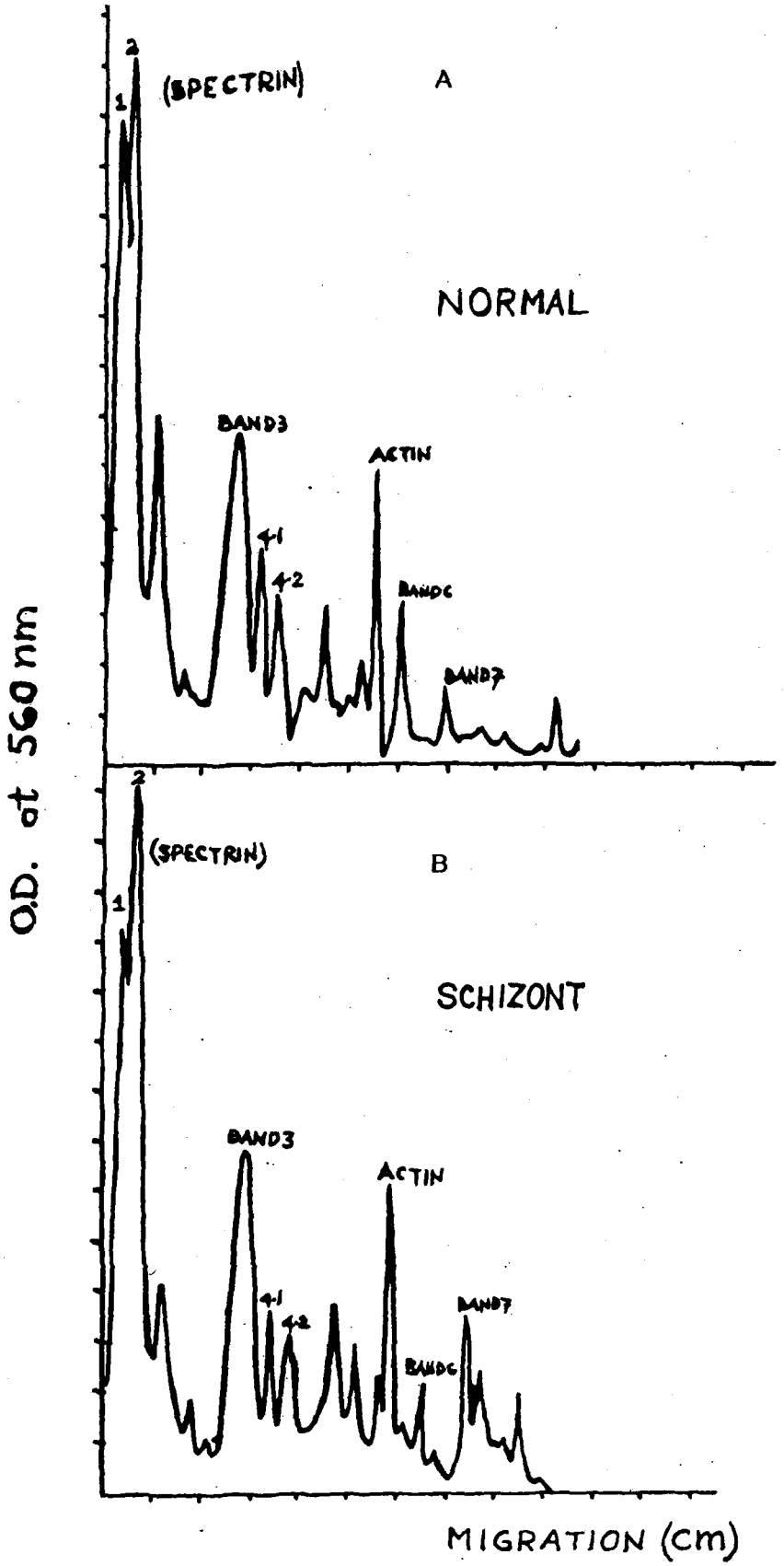



**Fig. 11:** Densitometric scans of Coomassie blue stained SDS-PAGE gels of erythrocyte membrane prepared from:
A - Normal monkey erythrocyte
B - Schizont-infected erythrocyte

To find out whether the new proteins are of parasite origin, the uninfected normal and infected erythrocytes, their ghosts and cytosols were electrophoresed on the SDS-polyacrylamide gels. Results are shown in Fig. 12. The proteins corresponding to the molecular weights of 163 kD, 90 kD, 55 kD and 23 kD were present in the whole infected erythrocytes but were completely absent in the normal cells. It may, therefore, be inferred that these proteins are of the parasite origin, but it is difficult to envisage whether these proteins have been inserted by the parasite into the host cell membrane, as reported earlier (108), or they originate from the parasite membrane contamination in the host cell membrane. The protein corresponding to molecular weight of 70 kD seems to be of host erythrocytes cytosol origin, as this protein is present in the host cell cytosol but is absent in the parasites.

**Identification of Neo Proteins by Crossed-Immunoelectrophoresis:**

To rule out the possibility of spectrin degradation in the parasitized erythrocytes the proteins separated in the first dimension on SDS-PAGE were electrophoresed, in the second dimension in the presence of antiserum raised against monkey erythrocytes spectrin in rabbits. Results of these experiments carried out on the trophozoite and schizont-infected erythrocytes are shown in Fig.13. Figure 13 clearly shows that anti-spectrin antibodies cross-reacted with spectrin bands only. No other band than these bands cross-reacted with these antibodies. These results confirm

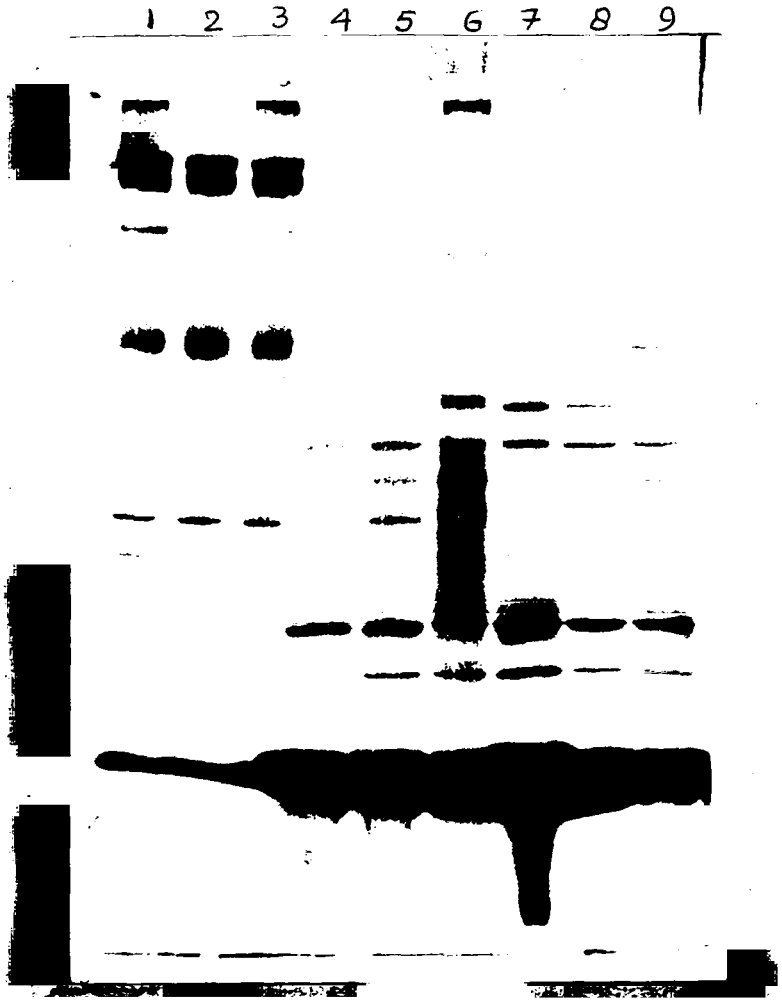



**Fig. 12:** SDS-polyacrylamide gel electrophoresis of erythrocyte membrane proteins and parasite containing red cells. Lane 1,2,3 contains normal, nonparasitized and schizont-infected red cell membrane proteins respectively. Lane 4,5,6 contain normal, nonparasitized and schizont-infected whole red cell proteins, respectively. Lane 7,8,9 have the respective cytosol of normal, nonparasitized and schizont-infected red cells.

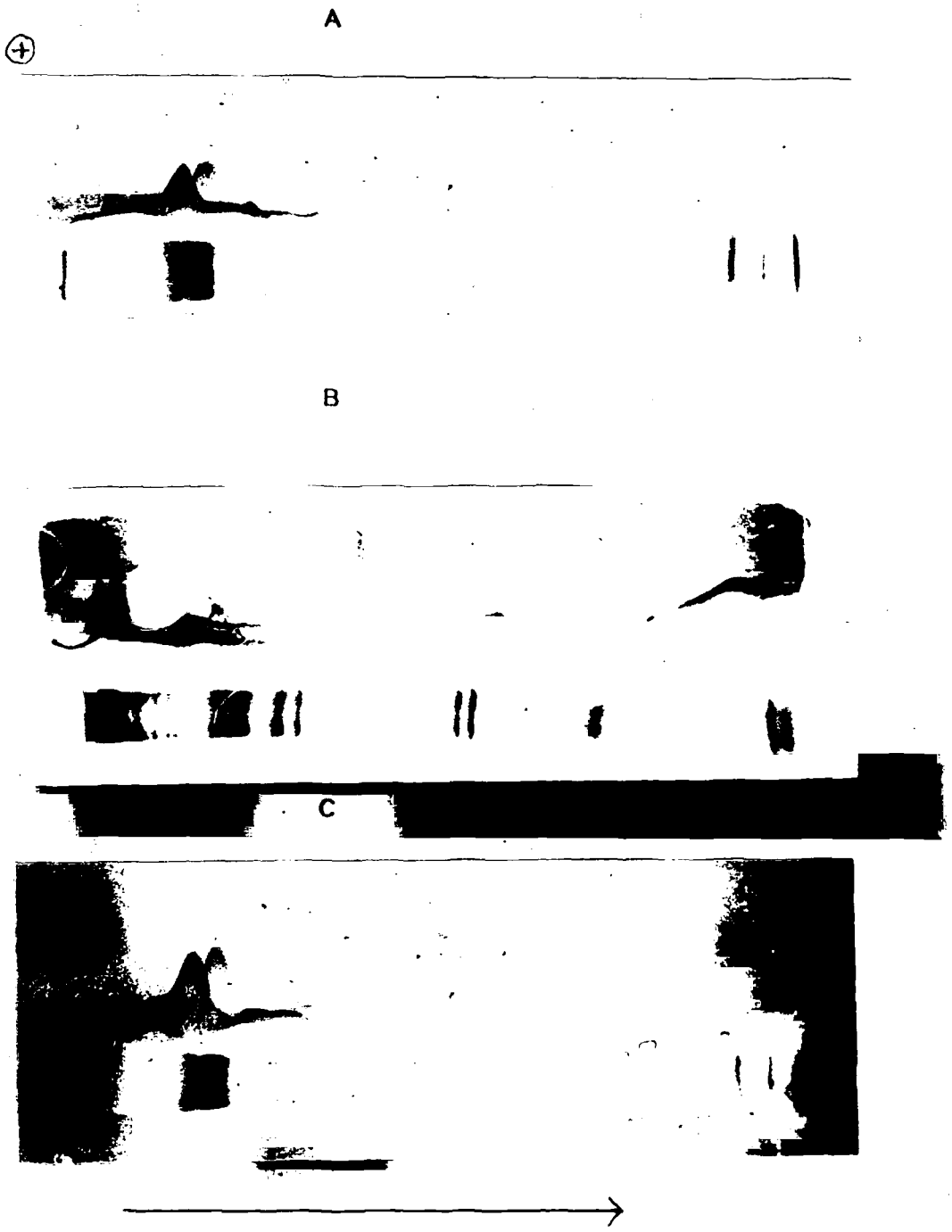



**Fig. 13:** Crossed immunoelectrophoretic analysis using antibody against spectrin. SDS-PAGE in the first dimension of membrane proteins (with arrows pointing in the anodic direction) was followed by immunoelectrophoresis in the second dimension (anode on top), against antispectrin-antiserum.

A - Normal monkey red cell membrane
B - *Trophozoite stage monkey red cell membrane*
C - Schizont stage monkey red cell membrane

the present finding that spectrin remains unaltered during infection of monkey erythrocytes with P.knowlesi.

These results are in contrast to the earlier findings of Wallach and Conley (107) but are consistent with the observation of Harvey Eisen (109) who showed that spectrin is not modified in the P.chabaudi - infected murine red cells.

**Analysis of Host Cell Membrane Sialoglycoproteins by Periodic Acid Schiff's (PAS) Staining:**

Sialoglycoproteins of monkey erythrocyte membrane were compared with the human erythrocyte membrane sialoglycoproteins after staining the SDS-PAGE with periodic acid Schiff's reagent. The monkey erythrocyte membrane proteins were different than of the human erythrocyte membrane proteins. The approximate molecular weight of the monkey erythrocyte membrane PAS-staining proteins are given in Table 3.

The effect of P.knowlesi-infection on the monkey erythrocyte membrane PAS-staining proteins was also determined at the trophozoite and schizont stage. These proteins were not altered at these stages of the infection (Fig. 14).

**Spectrin Tetramer-Dimer Equilibrium in Parasitized Red Cells:**

A typical elution profile from a Sepharose-CL-4B column of the $4^{o}C$ low-ionic strength extract of the membrane proteins is shown in Fig. 15. Gel electrophoretic analysis shows that

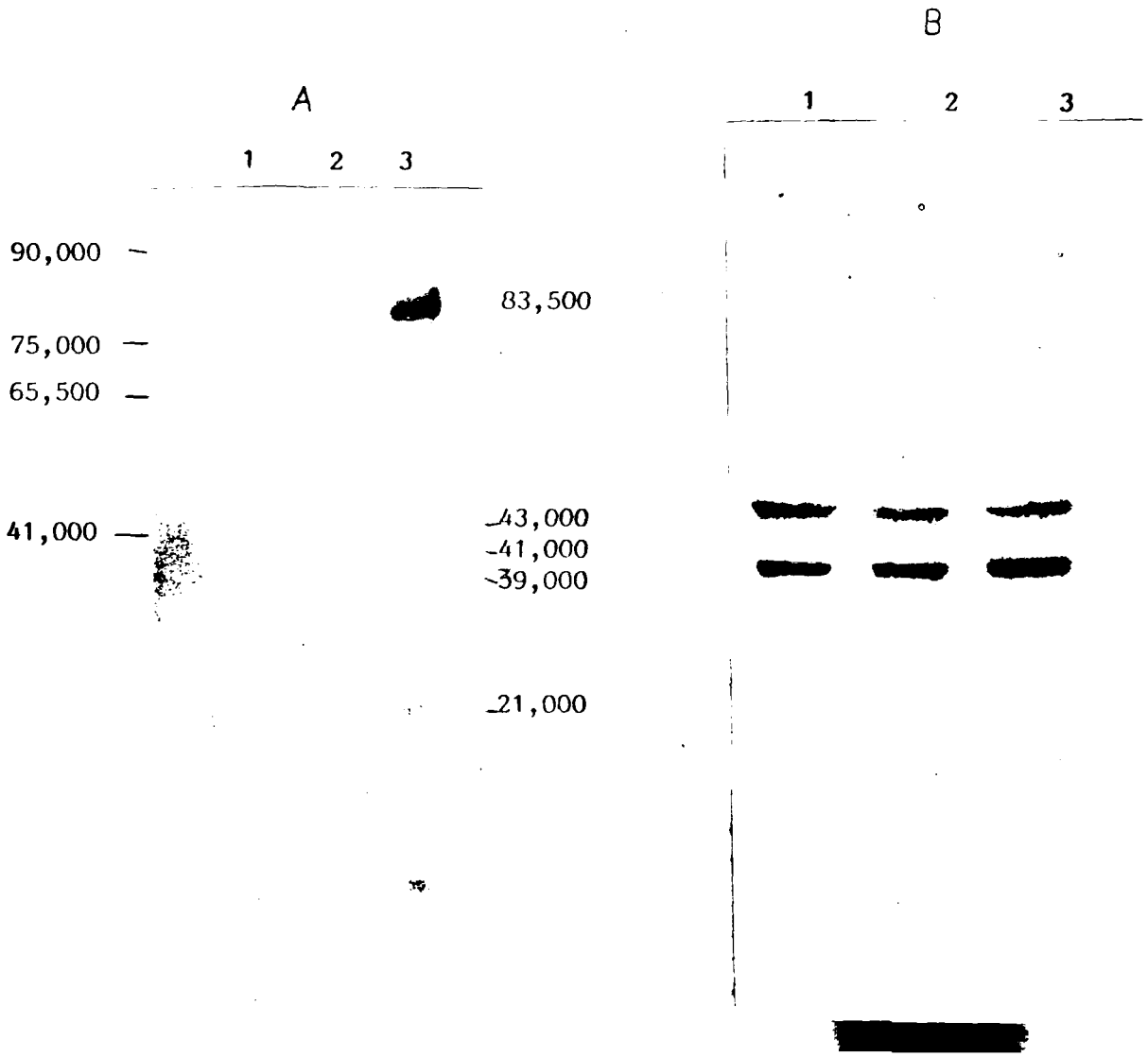



**Fig. 14:** Periodic-acid Schiff's staining of sialoglycoproteins of red cell membranes after SDS-PAGE.
A - Lane 1,2,3 contain normal monkey, trophozoite infected monkey red cell and human red cell membrane proteins respectively.
B - Lane 1,2,3 contain normal monkey, nonparasitized and schizont-infected monkey red cell membrane proteins respectively.

Table 3

Molecular Weights of Host Cell Glycoproteins

| Human Erythrocyte | | | Monkey Erythrocyte | | Schizont Infected Erythrocyte | |
|---|---|---|---|---|---|---|
| Name of Protein | | Apparent Mol. Weight | Name of Protein | Apparent Mol. Weight | Name of Protein | Apparent Mol. Weight |
| PAS 1 | | 83,500 | PAS $M_1$ | 90,000 | PAS $M_1$ | 90,000 |
| | | | PAS $M_2$ | 75,000 | PAS $M_2$ | 75,000 |
| | | | PAS $M_3$ | 65,500 | PAS $M_3$ | 65,000 |
| | X | 43,000 | | | | |
| PAS 2 | Y | 41,000 | PAS $M_4$ | 41,000 | PAS $M_4$ | 41,000 |
| | Z | 39,000 | | | | |
| PAS 3 | | 21,000 | | | | |

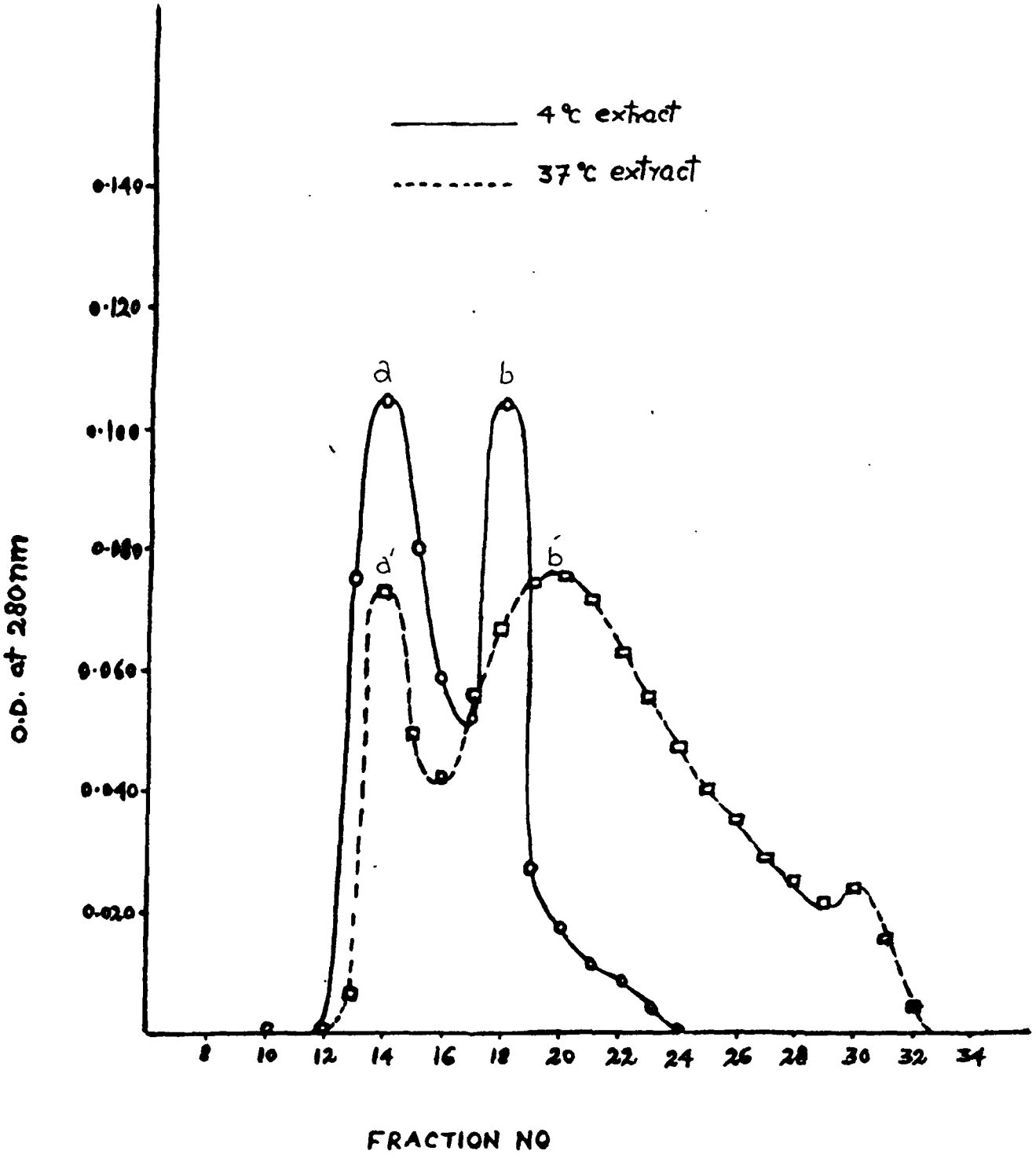



**Fig. 15:** Elution pattern of monkey erythrocyte cytoskelton from Sepharose CL-4B column. Samples ( 2 mg protein) loaded on the columns were extracted at $4^{o}C$ and $37^{o}C$ for 36 h and 30 min respectively.

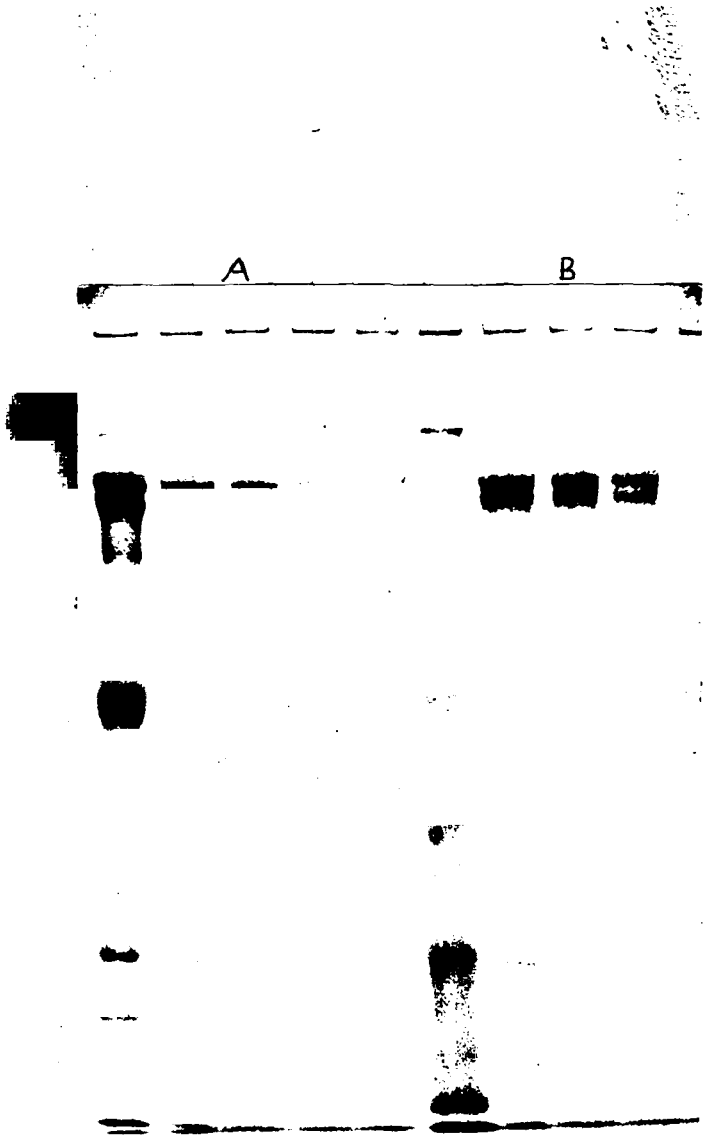



**Fig. 16:** SDS-PAGE of Sepharose CL-4B fractions (shown in Fig. 15) .
A - Lane 1 normal monkey ghost, Lane 2,3 contain peak a, Lane 4,5 contain peak b proteins, Lane 6 has marker proteins.
B - Lane 7,8 contain peak a' and Lane 9 has peak b' proteins.

both the prinicipal peaks are made up of spectrin. The leading peak (a) which corresponds to the void volume contains a mixture of oligomeric species, whereas the second peak (b) consists entirely of spectrin tetramer. Peak (a) also contains actin as is evident from Fig. 16. The heated sample of the low ionic strength extract has two major peaks and one very small peak. The second peak was broad and shifted (Fig.15 ). The broadening of the peak showed that it was a mixture of two species of spectrin namely tetramer and dimer. Peak recovered in the broadening zone was free from actin. The first peak contained actin, but it was smaller than that in $4^{o}C$ extract.These elution profiles remained unaltered when spectrin-actin were extracted from the membranes of Schizont-infected erythrocytes in identical condition (Fig.17). These results indicate that spectrin dimer-tetramer equilibrium is not affected by the presence of malarial parasites whithin the monkey erythrocytes.

**Extractability of Cytoskeleton from Host Cell Membrane:**

Red cell membrane cytoskeleton was extracted from parasitized and normal red cells by low ionic strength buffer under identical conditions. The extractability of the cytoskeleton from the membranes of the parasitized red cells was similar to that of the normal cells (Table 4). This indicates that cytoskeleton-membrane bilayer interactions are not significantly altered in the infected erythrocytes.

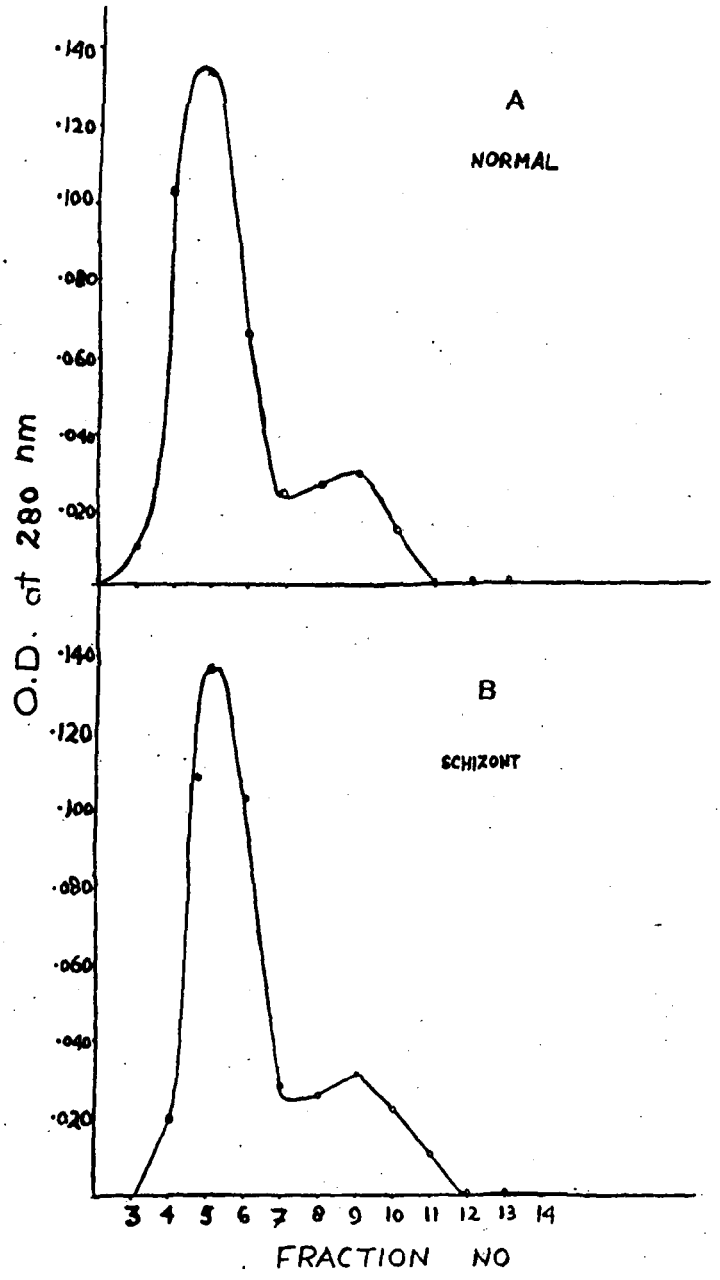



**Fig. 17:** Elution pattern of 4°C cytoskeleton extract from Sepharose CL-4B column:

A - From monkey red cell
B - From schizont-infected red cell

Table 4

Cytoskeleton Extractability from Host Cell Membrane.

| Temperature 0-4°C for 36 hrs | |
|---|---|
| Source of Membrane | % Extractability |
| Normal | 14.80 (± 1.28) |
| Parasitized | 13.22 (± 1.80) |

# DISCUSSION

Deformation and mechanical properties of the red cells are controlled by the interaction of the membrane bilayer with the underlying membrane skeleton. Any attempt to deform the red cells is resisted by the membrane skeleton which is a meshwork of three major and few minor proteins, of which spectrin is the major structural constituent. Therefore, it is implied for the malarial parasite to enter in the red cell, the external parasite must alter the membrane skeleton-bilayer interaction to facilitate its entry into the cells.

Earlier studies have shown that the cytoskeleton protein spectrin, is degraded in the infected cells (107, 101, 92). This observation has generally been used to explain the altered cytoskeleton-bilayer interaction in the infected cells. However, no precaution has been taken in these studies to eliminate the possibilities of the host cell membrane protein degradation by the parasite proteases which will be released during lysis of the cells in the process of membrane preparation. It is likely that these proteases degrade the host cell membrane proteins during the work-up of the membrane, rather than the protein degraded by the parasite within the cell. To examine this possibility, the structure of spectrin was studied in rhesus monkey red cells infected with different developmental stages of P. knowlesi.

Results discussed in the preceding section clearly shown that spectrin to band 3 ratio remains virtually unaltered after

infection of the erythrocyte with the malarial parasite. That spectrin is not altered in the infected cell was further confirmed by immunochemical analysis. Spectrin was purified from normal monkey red cells and antibodies to this protein was raised in rabbits. The anti-sera so-obtained was used to identify proteins related to spectrin, if there is any, by crossed-immunoelectrophoresis. Results of these studies have revealed that the spectrin-specific antibodies react with no other protein than spectrin. This further suggests that spectrin in infected cells is not structurally modified.

To further confirm this finding, the spectrin tetramer-to dimer ratio was studied in the infected cells, as this ratio has been shown to change when spectrin undergoes structural abnormality (110, 111). In the infected cells, the spectrin tetramer-to-dimer ratio was similar to that observed in the normal rhesus monkey erythrocytes. It may, therefore, be inferred that spectrin structure is not altered by the parasite either during invasion or during its development in the host erythrocyte.

To investigate whether there is any change in the intensity of interaction between the membrane bilayer and membrane skeleton, the extractability of the cytoskeleton from the infected and normal erythrocyte ghosts was analyzed, since it should grossly depend on these interactions. As there was no change in the extractability of the cytoskeleton after infection, it may be inferred that the

interactions of the membrane bilayer with membrane skeleton are probably not affected in the infected cells.

This study shows that the major membrane skeletal protein, spectrin, is not structurally modified by the malarial parasite. Therefore, for its entry, the external parasite should employ some other mechanisms than the spectrin degradation, to alter the host erythrocyte cytoskeleton. It may secrete some proteins which could span the membrane bilayer as well as can interact with the membrane skeleton. These interactions in turn could then modify the host cell membrane deformability. Such proteins have already been shown to be secreted by P.falciparum during invasion (112). Alternatively, the external parasite can induce expansion of the host cell membrane bilayer by secreting lipids on the membrane surface which, consequently would affect the membrane bilayer-cytoskeleton interactions. That Plasmodium secretes lipids during invasion has recently been demonstrated in case of P.falciparum (113) and earlier in case of P.knowlesi (89). Besides these possibilities, it may also be envisaged that the external parasite could secrete some proteolytic enzymes into the host cell cytosol by some as yet unknown mechanism which could result in degradation of some strategic proteins, like ankyrin. Finally, the parasite could secrete some water soluble proteins which may compete for binding with cytoskeleton proteins. However, the present study is not sufficient to distinguish between these possibilities.

The present study has also impact on the mechanisms that regulate the transbilayer movement of phospholipids in the erythrocyte membrane. In normal erythrocytes, it is believed that these movements are controlled by the interaction of the aminophospholipids with spectrin (54). In cases where spectrin becomes abnormal, the aminophospholipids which are normally located almost exclusively in the inner-monolayer tend to partially migrate to the outer surface. In malaria-infected cells it has earlier been shown that the phospholipid movement across the membrane bilayer is significantly enhanced. Since spectrin in infected erythrocytes is not structurally altered, it would seem that the spectrin-phospholipid interactions in the erythrocytes have no primary role in regulating the transmembrane movements of the various phospholipids within the membrane bilayer.

# BIBLIOGRAPHY

1. Tanford, C. (1978). The Hydrophobic Effect: Formation of Micelles and Biological Membranes. John Wiley and Sons, New York.

2. Danielli, J.F. and Davson, H. (1935). The permeability of thin films. Journal of Cellular Comparative Physiology, 5, 495-508.

3. Chapman, D. (1975). Phase transitions and fluidity characteristics of lipids cell membrane. Quaterly Reviews of Biophysics 8, 135-235.

4. Singer, S.J. and Nicolson, G.L. (1972). The fluid mosaic model of the structure of cell membranes. Science, 175, 720-731.

5. Nicolson, G.L. (1976). Transmembrane control of the receptors on normal and tumor cells. Biochim. Biophys. Acta 457, 57-108.

6. Levine, H., III, Sahyoun, N.E. and Cuatrecasas, P. (1982). Properties of rat erythrocyte membrane cytoskeltal structure produced by Digitonin extraction: Digitonin-insoluble/3-adrenergic receptor adenylate cyclase, and cholera toxin substrate. Journal of Membrane Biology, 64, 225-231.

7. Volsky, D.J. and Loyter, A. (1978). Role of $Ca^{++}$ in virus-induced membrane fusion. J. Cell Biol., 78, 465-479.

8. Hardy, B., Bensch, K.G. and Schrier, S.L. (1979). Spectrin rearrangement early in erythrocyte ghost endocytosis. J. Cell Biol., 82, 654-663.

9. Bretscher, M.S. (1972). Phosphatidylethanolamine: Differential labeling in intact cells and cell ghosts of human erythrocytes by a membrane impermeable reagent. J. Mol. Biol. 71, 523-528.

10. Lux, S.E. and Glader, B.E. (1981). Disorders of the red cell membrane. In Hematology of infancy and Childhood (ed. D.G. Nathan and I.S. Oshi), pp, 456-565. Philadelphia:Saunders.

11. Gratzer, W.B. (1982). The cytoskeleton of red blood cell. In: Muscle and nonmuscle motility, Vol.2, (ed. Stracher, A), pp 37-124, Academic press, New York.

12. Ralston, G.B. (1976). Physico-chemical characterization of the spectrin tetramer from bovine erythrocyte membranes. Biochim. Biophys. Acta, 455, 163-172.

13. Ungewickell, E. and Gratzer, W.B.(1978). Self association of human spectrin. A thermodynamic and kinetic study. Eur. J. Biochem., 88, 379-385.

14. Goodman, S.R. and Weidner, S.A. (1980). Binding of spectrin $\alpha_2$-$\beta_2$ tetramers to human erythrocyte membranes. J. Biol. Chem., 255, 8082-8086.

15. Liu, S.C., Palek, J. (1980). Spectrin tetramer-dimer equilibrium and the stability of erythrocyte membrane cytoskeletons. Nature, 285, 586-88.

16. Liu, S.C.,Windisch, P., Kim, S. and Palek, J (1984). Oligometric states of spectrin in normal erythrocyte membranes: biochemical and electron microscopic studies. Cell, 37, 587-594.

17. Gratzer, W.B. and Beaven, G.H. (1975). Properties of the high molecular weight protein (spectrin) from human erythrocyte membranes. Eur. J. Biochem., 58, 403-409.

18. Ralston, G.B. and Dunbar, J.C. (1979). Salt and temperature dependent conformation changes in spectrin from human erythrocyte membranes. Biochim. Biophys. Acta, 579, 20-30.

19. Calvert, R., Ungewickell, E. and Gratzer, W.B. (1980). A conformational study of human spectrin. Eur. J. Biochem., 107, 363-367.

20. Elgsacter, A. (1978). Human spectrin. I. A classical light scattering study. Biochim. Biophys. Acta, 536, 235-244.

21. Calvert, R., Bennett, P. and Gratzer, W.B. (1980). Properties and structural role of the subunits of human spectrin. Eur. J. Biochem., 107, 355-361.

22. Shotton, P.M., Burke, B.E. and Branton, D. (1979). Molecular structure of human erythrocyte spectrin: biophysical and electron miscroscopic studies. J. Mol. Biol., 131, 303-329.

23. Speicher, D.W. and Marchesi, V.T. (1984). Erythrocyte spectrin is composed of many homologous triple helical segments. Nature, 311, 177-180.

24. Harris, H.W. Jr. & Lux, S.E. (1980). Sturcutral characterization of the phosphorylation sites of human erythrocyte spectrin. J. Biol. Chem., 255, 11512-11520.

25. Pinder, J.C. and Gratzer, W.B. (1983). Structural and dynamic states of actin in the erythrocyte. J. Cell Biol. 96, 768-775.

26. Sheetz, M.P., Painter, R.G. and Singer, S.J. (1976). Relationships of the spectrin complex of human erythrocyte membranes to the actomyosins of muscle cells. Biochemistry, 15, 4486-4492.

27. Shen, B.W., Josephs, R. and Steck, T.L. (1986). Ultrastructure of the intact skeleton of the human erythrocyte membrane. J. Cell Biol. 102, 997-1006.

28. Tyler, J.M., Reinhardt, B.N. and Branton, D. (1980). Associations of erythrocyte membrane proteins: Binding of purified bands 2.1 and 4.1 to spectrin. J. Biol. Chem.,255, 7034-7039.

29. Ohanian, V., Wolfe, L., John, K., Pinder, J., Lux, S. and Gratzer, W. (1984). Analysis of the ternary interactions of the red cell membrane skeletal proteins spectrin, actin and 4.1. Biochemistry, 23, 4416-4420.

30. Mueller, T., Jackson, C., Doktor, M. and Morrison, M. (1987). Membrane skeletal alterations during in vivo mouse red cell aging: Increase in the band 4.1a:4.1b ratio. J. Clin. Invest. 79, 492-499.

31. Conboy, J., Mohandas, N., Tchernia, G. and Kan, Y. (1986). Molecular basis of hereditary elliptocytosis due to protein 4.1 deficiency. New Eng. J. Med., 315, 680-685.

32. Korsgren, C. and Cohen, C.M. (1986). Purification and properties of human erythrocyte band 4.2. J. Biol. Chem., 261, 5536-5543.

33. Siegel, D.L. and Branton, D. (1985). Partial purification and characterization of an actin-bundling protein, band 4.9, from human erythrocytes. J. Cell Biol., 100, 775-785.

34. Fowler, V.M. and Bennett, V. (1984). Erythrocyte membrane tropomyosin. Purification and properties. J. Biol. Chem., 259, 5978-5989.

35. Kirkpatrick, F.H. and Sweeney, M.L. (1980). Cytoplasmic and membrane bound erythrocytic myosin(s). Fed. Proc. 39, 2049a.

36. Fairbanks, G., Steck, T.L.and Wallach, D.F.H. (1971). Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry, 10, 2606-2617.

37. Lux, S.E. (1983). Disorders of the red cell membrane skeleton:Hereditary spherocytosis and heriditary elliptocytosis. In Metablic Basis of Inherited Disease, 5th Edn. (ed. J. Stanbury), pp 1573-1605. New York: McGraw Hill.

38. Morrison, M., Mueller, T.J. and Edwards, H.H. (1981). Protein architecture of the erythrocyte membrane. In: The Function of Red Blood Cells: Erythrocyte Pathobiology. ed. Wallach, D.F.H., pp 17-34, Alan R. Liss Inc. New York.

39. Tanner, M. (1983). Erythrocyte membrane structure and function. Ciba Foundation Symposium 94, 15-23.

40. Steck, T.L. (1978). The band 3 protein of the human red cell membrane: A review. J. Supramol. Struct., 8, 311-324.

41. Toon, M.R., Dorogi, P.L., Lukacovic, M.F. and Solomon, A.K. (1985). Binding of DTNB to band 3 in the human red cell membrane. Biochim. Biophys. Acta 818, 158-170.

42. Kaul, R.K., Prasanna Murthy, S.N., Reddy, A.G., Steck, T.L. and Kohler, H. (1983). Amino acid sequence of the N$\alpha$-terminal 201 residues of human erythrocyte membrane band 3. J. Biol. Chem., 258, 7981-7990.

43. Jennings, M.L., Mohaghan, R., Douglas, S.M. and Nicknish, J.S. (1985). Functions of extracellular lysine residues in the human erythrocyte anion transport protein. J. Gen. Physiol. 86, 653-669.

44. Jones, M.N. and Nickson, J.K. (1981). Monosaccharide transport proteins of the human erythrocyte membrane. Biochim. Biophys. Acta, 650, 1-20.

45. Lowe, A.G. and Walmsley, A.R. (1987). A single half-tunrover of the glucose carrier of the human erythrocyte. Biochim. Biophys. Acta, 903, 547-550.

46. Cairns, M.T., Elliot, D.A., Scudder, P.R. and Baldwin, S.A. (1984). Proteolytic dissection of the human erythrocyte glucose transporter. Biochem. J., 221, 179-188.

47. Bennett, V. and Branton, D. (1977). Selective association of spectrin with the cytoplasmic surface of human erythrocyte plasma membranes. J. Biol. Chem., 252, 2753-2763.

48. Morrow, J.S., Speicher, D.W., Knowles, W.J., Hsu, C.J., Marchesi, V.T. (1980). Identification of functional domains of human erythrocyte sepctrin. Proc. Natl. Acad. Sci. USA, 77, 6592-6596.

49. Sheetz, M.P. (1979). Integral membrane protein interaction with triton cytoskeletons of erythrocytes. Biochim. Biophys. Acta, 557, 122-134.

50. Ohanian, V. and Gratzer, W. (1984). Preparation of red-cell-membrane cytoskeletal constituents and characterization of protein 4.1. Eur. J. Biochem., 144, 375-379.

51. Cohen, C.M. and Foley, S.F. (1984). Biochemical characterization of complex formation by human erythrocyte spectrin, protein 4.1, and actin. Biochemistry, 23, 6091-6098.

52. Haest, C.W.M. (1982). Interactions between membrane skeleton proteins and the intrinsic domain of the erythrocyte membrane. Biochim. Biophys. Acta, 694, 331-352.

53. Bretscher, M.S. (1972). Phosphatidylethanolamine: Differential labelling in intact cells and cell ghosts of human erythrocytes by a membrane-impermeable reagent. J. Mol. Biol. 71, 523-528.

54. Heast, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Spectrin as a stabilizer of phospholipid asymmetry in the human erythrocyte membrane. Biochim. Biophys. Acta, 509, 21-23.

55. Chiu, D., Lubin, B., Roelofsen, B. and van Deenen, L.L.M. (1981). Sickled erythrocytes accelerate clotting in vitro: An effect of abnormal membrane lipid asymmetry. Blood, 58, 398-401.

56. Mombers, C., Verkleij, A.J., de Gier, J. and van Deenen, L.L.M. (1979). The interaction of spectrin-actin and synthetic phospholipids. II. The interaction with phosphatidylserine. Biochim. Biophys. Acta, 603, 52-62.

57. Mombers, C., de Gier, J., Demel, R.A. and van Deenen, L.L.M. (1980). Spectrin-phospholipid interaction-A monolayer study. Biochim. Biophys. Acta, 603, 52-62.

58. Sato, S.B. and Ohnishi, S.I. (1983). Interaction of a peripheral protein of the erythrocyte membrane, band 4.1, with phosphatidylserine-containing liposomes and erythrocyte inside-out vesicles. Eur. J. Biochem., 130, 19-25.

59. Op den Kamp, J.A.F. (1979). Lipid asymmetry in membranes. Ann. Rev. Biochem., 48, 47-71.

60. van Deenen, L.L.M. (1981). Topology and dynamics of phospholipids in membranes. FEBS Letter, 123, 1-15.

61. Dodge, J.T. and Phillips, G.B. (1967). Composition of phospholipids and of phospholipid fatty acids and aldehydes in human red cell. J. Lipid Res., 8, 667-675.

62. Shohet, S.B. (1976). Mechanisms of red cell membrane lipid renewal. In: Membrane and diseases (eds. Bolis, L., Hoffman, J.F. and Leaf, A.), pp. 61-74, Raven Press, New York.

63. Williamson, P., Bateman, J., Kozarsky, K., Mattocks, K., Hermanowicz, N., Choe, H.R. and Schlegel, A. (1982). Involvement of spectrin in the maintenance of phase state asymmetry in the erythrocyte membrane. Cell, 30, 725-733.

64. Zwall, R.F.A., Comfurius, P. and van Deenen, L.L.M. (1977). Membrane asymmetry and blood coagulation. Nature, 268, 358-360.

65. Sandermann, H., Jr. (1978). Regulation of membrane enzymes by lipids. Biochim. Biophys. Acta, 515, 209-237.

66. Girand, F., Zali, H.M., Chailley, B. and Mazet, F. (1984). Changes in morphology and in polyphosphoinositide turnover of human erythrocytes after cholesterol depletion. Biochim. Biophys. Acta, 778, 191-200.

67. Fisher, K.A. (1976). Analysis of membrane halves: cholesterol. Proc. Natl. Acad. Sci. USA, 73, 173-177.

68. Schroeder, F. (1981). Use of a fluorescent sterol to probe the transbilayer distribution of sterols in biological membranes. FEBS Letter, 135, 127-130.

69. Hale, J.E. and Schroeder, F. (1982). Asymmetric transbilayer distribution of sterol across plasma membranes determined by fluorescence quenching of dehydroergosterol. Eur. J. Biochem., 122, 649-661.

70. Rothman, J.E. and Lenard, J. (1977). Membrane asymmetry-The nature of membrane asymmetry provides clues to the puzzle of how membranes are assembled. Science, 195, 743-753.

71. Miller, L.H. (1977). Hypothesis on the mechanism of erythrocyte invasion of malaria merozoites. Bull. W.H.O., 55, 157-162.

72. Aikawa, M., Miller, L.H., Johnson, J. and Rabbege, J. (1978). Erythrocyte entry by malarial parasites. A moving junction between erythrocyte and parasite. J. Cell Biol., 77, 72-82.

73. McGhee, R.B. (1953). The infection by Plasmodium lophurae of duck erythrocytes in the chicken embryo. J. Exp. Med., 97, 773-782.

74. Miller, L.H., Devorak, J.A., Shiroishi, T. and Durocher, J.R. (1973). Influence of erythrocyte membrane components on malaria merozoite invasion. J. Exp. Med., 138, 1597-1601.

75. Miller, L.H., Mason, S.J., Dvorak, J.A., Mc Ginniss, M.H. and Rothman, I.K. (1975). Erythrocyte receptors for (Plasmodium knowlesi)malaria: Duffy blood group determinants. Science, 189, 561-563.

76. Pasvol, G. and Wilson, R.J.M. (1982). The interaction of malaria parasites with red blood cells. British Med. Bull., 38, 133-140.

77. Jungery, M. (1985). Studies on the biochemical basis of the interaction of the merozoite of Plasmodium falciparum and the human red cell. Trans. Roy. Soc. Trop. Med. Hyg., 79, 591-597.

78. Okoye, V.C.N. and Bennett, V. (1985). Plasmodium falciparum malaria: Band 3 as a possible receptor during invasion of human erythrocytes. Science, 227, 169-171.

79. McLaren, D.J., Bannister, L.H., Trigg, P.I. and Butcher, G.A. (1979). Freeze studies on the interaction between the malaria parasite and the host-erythrocyte in Plasmodium knowlesi infection. Parasitology, 79, 125-139.

80. Aikawa, M., Miller, L.H., Rabbege, J.R. and Epstein, N. (1981). Freeze fracture study on the erythrocyte membrane during malarial parasite invasion. J. Cell Biol. 91, 55-62.

81. Kidson, C., Lamont, G., Saul, A. and Nurse, G.T. (1981). Ovalocytic erythrocytes from Melanesians are resistant to invasion by malaria parasites in culture. Proc. Natl. Acad. Sci. U.S.A., 78, 5829-5832.

82. Dluzewski, A.R., Rangachari, K., Wilson, R.J.M. and Gratzer, W.B. (1983). Cytoplasmic requirement of red cells for invasion by malarial parasites. Mol Biochem. Parasitol., 9, 145-160.

83. Trager, W., Rudzinska, M.A. and Bradbury, P.C. (1966). The fine structure of Plasmodium falciparum and its host erythrocytes in natural malarial infections in man. Bull W.H.O., 35, 883-885

84. Allred, D.R., Gruenberg, J.E. and Sherman, I.W. (1986). Dynamic rearrangements of erythrocyte membrane internal architecture induced by infection with Plasmodium falciparum. J. Cell Sci., 81, 1-16.

85. Udeinya, I.J., Schmidt, J.A., Aikawa, M., Miller, L.H. and Green, I. (1981). Falciparum malaria-infected erythrocytes specifically bind to cultured human endothelial cells. Science, 213, 555-557.

86. Aikawa, M., Udeinya, I.J., Rabbege, J., Dayan, M., Leech, J.H., Howard, R.J. and Miller, L.H. (1985). Structural alteration of the membrane of erythrocytes infected with Plasmodium falciparum. J. Protozool., 32, 424-429.

87. Seed, T.M. and Krier, J.P. (1972). Plasmodium gallinaceum: Erythrocyte membrane alterations and associated plasma changes induced by experimental infection. Proc. Helminthol. Soc. Washington, 39, 387-411.

88. Cooper, G.W. and Miller, L.H. (1974). Propanoic acid-ferric oxide hydrosols. Differential cell surface binding and its relation to membrane lipid. J. Histochem. Cytochem., 22, 856-867.

89. Gupta, C.M. and Mishra, G.C. (1981). Transbilayer phospholipid asymmetry in Plasmodium knowlesi infected host cell membrane. Science, 212, 1047-1049.

90. Joshi, P., Dutta, G.P. and Gupta, C.M. (1987). An intracellular simian malarial parasite (Plasmodium knowlesi) induces stage-dependent alterations in membrane phospholipid organization of its host erythrocyte. Biochem. J., 246, 103-108.

91. Schwartz, R.S., Olson, J.A., Raventos-Suarez, C., Yee, M., Heath, R.H., Lubin, B. and Nagel, R.L. (1987). Altered plasma membrane phospholipid organization in Plasmodium falciparum - infected human erythrocytes. Blood, 69, 401-407.

92. Sherman, I.W. and Jones, L.A. (1979). Plasmodium lophurae: Membrane proteins of erythrocyte-free plasmodia and malaria-infected red cells. J. Protozool., 26, 489-501.

93. Gupta, C.M. (1985). A reappraisal of red cell membrane changes in malarial infection. Perspectives in Parasitology, 1, 205-225.

94. Lorand, L., Bijerrum, O.J., Hawkins, M., Krentz, L.L. and Siefring, G.E., Jr. (1983). Degradation of transmembrane proteins in $Ca^{++}$ enriched human erythrocytes. J. Biol. Chem., 258, 5300-5305.

95. Howard, R.J. (1982). Alterations in the surface membrane of red blood cells during malaria. Immunological Reviews, 61, 67-107.

96. Aley, S.B., Barnwell, J.W., Daniel, W. and Howard, R.J. (1984). Knob-positive and knob-negative Plasmodium falciparum differ in expression of strain specific malarial antigen on the surface of infected erythrocytes. J. Exp. Med., 160, 1585-1590.

97. Brown, K.N. (1977). Antigenic variation in malaria.In: Immunity of blood parasites of animals and man, (eds. Miller, L.H., Pino, J.A. and McKelvey, J.J. Jr.), pp 5-25, Plenum Press, New York/London.

98. Kutner, S., Breur, W.V., Ginsburg, M., Aley, S.B. and Cabantchik, Z.I. (1985). Characterization of permeation pathways in the plasma membrane of human erythrocytes infected with early stages of Plasmodium falciparum : Association with parasite development. J. Cell. Physiol., 125, 521-527.

99. Sherman, I.W. (1989). Biochemistry of Plasmodium (Malarial Parasites). Microbiological Reviews, 43, 453-495.

100. Weidekamm, E., Wallach, D.F.H., Lin, P.S. and Hendricks, J. (1973). Erythrocyte membrane alterations due to infection with Plasmodium berghei. Biochim. Biophys. Acta, 323, 539-546.

101. Yuthavong, Y., Wilairat, P., Panijpan, B., Potiwan, C. and Beale, G.H. (1979). Alterations in membrane proteins of mouse erythrocytes infected with different species and strains of malaria parasites. Comp. Biochem. Physiol., 63b, 83-85.

102. Lorand, L., Bjerrum, O.J., Hawkins, M., Lowe-Krentz, L. and Siefring, G.E., Jr. (1983). Degradation of transmembrane proteins in $Ca^{++}$-enriched human erythrocytes. J. Biol.Chem., 5300-5305.

103. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature (Lond), 277, 680-685.

104. Vander Jagt, D.L., Intress, C., Heidrich, J.E., Mrema, J.E.K., Rieckmann, K.H. and Heidrich, H.G. (1982). Marker enzymes of Plasmodium falciparum and human erythrocytes as indicators of parasite purity. J. Parasitol., 68, 1068-1071.

105. Banyal, H.S., Mishra, G.C., Gupta, C.M. and Dutta, G.P. (1981). Involvement of malarial proteases in the interaction between the parasite and host erythrocyte in Plasmodium knowlesi infections. J. Parasitol., 67, 623-626.

106. Singhal, A., Bali, A. and Gupta, C.M. (1986). Antibody-mediated targetting of liposomes to erythrocytes in whole blood. Biochim. Biophys. Acta, 515, 367-394.

107. Wallach, D.F.H. and Conley, M. (1977). Altered membrane proteins of monkey erythrocytes infected with simian malaria. J. Mol. Med., 2, 119-136.

108. Schmidt-Ullrich, R. and Wallach, D.F.H. (1978). Plasmodium knowlesi - induced antigens in membranes of parasitized rhesus monkey erythrocytes. Proc. Natl. Acad. Sci. U.S.A., 75, 4949-4953.

109. Eisen, H. (1977). Purification of intracellular forms of Plasmodium chabaudi and their interactions with erythrocyte membrane and serum albumin. Bull. W.H.O., 55, 333-338.

110. Palek, J., Liu, S-C, Liu, P-Y, Prchal, J., Castleberry, R.P. (1981). Altered assembly of spectrin in red cell membranes in hereditary pyropoikilocytosis. Blood, 57, 130-139.

111. Coetzer, T. and Zail, S. (1982). Spectrin tetramer-dimer equilibrium in hereditary elliptocytosis. Blood, 59, 900-905.

112. Sam-Yellowe, T.Y., Shio, H. and Perkins, M.E. (1988). Secretion of Plasmodium falciparum rhoptry protein into the plasma membrane of host erythrocytes. J. Cell Biol., 106, 1507-1517.

113. Mikkelsen, R.B., Kamber, M., Wadwa, K.S., Linn, P-S and Schimidt, Ullrich, R. (1988). The role of lipids in Plasmodium falciparum invasion of erythrocytes: A coordinated biochemical and microscopic analysis. Proc. Natl. Acad. Sci. USA, 85, 5956-5960.

114. Beumelle, B.D., Vial, H.J. and Bienvenne, A. (1988). Enhanced transbilayer mobility of phospholipids in malaria-infected monkey erythrocytes: A spin-label study. J. Cell. Physiol. 135, 94-100.

115. Gratzer, W.B. (1983). The cytoskeleton of the red blood cell. In Muscle and Nonmuscle Mobility Vol.2. (ed. Stracher) pp. 37-124. New York Academic Press.

116. Goodman, S.R. and Schiff, K. (1983). The spectrin membrane skeleton of normal and abnormal human erythrocytes. American Journal of Physiology, 244, C124-41.

117. Gardner, K. and Bennett, V. (1988). In: The Red Cell Membrane: Structure, Function, and Clinical Implications (eds. Agre, P. and Parker, J.C.) Marcel Dekker Inc, New York, NY.